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**PATENT APPLICATION**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

In re application of

Docket No: Q52816

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155

Group Art Unit: 1616

Confirmation No.: 5866

Examiner: Sabiha N. Qazi

Filed: December 29, 1998

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

**SUBMISSION OF APPEAL BRIEF**

**MAIL STOP APPEAL BRIEF - PATENTS**

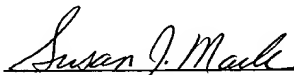
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Submitted herewith please find an Appeal Brief. A check for the statutory fee of \$340.00 is attached. The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account. A duplicate copy of this paper is attached.

Respectfully submitted,

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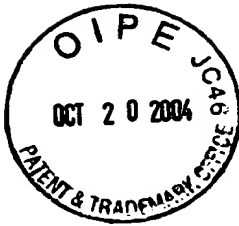
  
Susan J. Mack  
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WASHINGTON OFFICE

**23373**

CUSTOMER NUMBER

Date: October 20, 2004



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**APPEAL BRIEF UNDER 37 C.F.R. § 41.37**

**MAIL STOP APPEAL BRIEF - PATENTS**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

In accordance with the provisions of 37 C.F.R. § 41.37, Appellant submits the following:

The following comprises the Appellant's Brief on Appeal from the Office Action dated April 20, 2004, wherein claims 3 and 4 were finally rejected.

Appellant's Notice of Appeal was filed on August 20, 2004. Therefore, the present Appeal Brief is timely filed.

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APPEAL BRIEF UNDER 37 C.F.R. § 41.37  
U.S. Appln. No.: 09/214,155

**I. REAL PARTY IN INTEREST**

The real party in interest is Teijin Limited, a Japanese Body Corporate.

**II. RELATED APPEALS AND INTERFERENCES**

There are no other appeals or interferences known to Appellants, the Appellants' legal representative, or Assignee which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

APPEAL BRIEF UNDER 37 C.F.R. § 41.37  
U.S. Appln. No.: 09/214,155

### **III. STATUS OF CLAIMS**

Claims 3 and 4 are pending in the application, and their status is as follows:

Claims 3 and 4 are rejected and are the subject of this appeal.

#### **IV. STATUS OF AMENDMENTS**

On December 22, 2003, Appellants filed an Amendment Under 37 C.F.R. § 1.111 in response to the non-final Office Action mailed August 22, 2003. That Amendment was entered. There has not been an amendment filed in response to the April 20, 2004, final Office Action. Accordingly, the claims stand as presented in the Amendment filed December 22, 2003 (see attached Appendix).

**V. SUMMARY OF THE CLAIMED SUBJECT MATTER**

The claimed subject matter relates to novel 1, 25-dihydroxy-2-methylvitamin D<sub>3</sub> compounds having unexpectedly superior properties, and methods for producing the compounds. Specification, page 1, lines 8-10.

Claim 3 provides a 1,25-dihydroxy-2-methylvitamin D<sub>3</sub> compound, wherein the compound is (i) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\beta$ -methyl-3 $\beta$ -vitamin D<sub>3</sub>; (ii) (20S)-1 $\beta$ , 25-dihydroxy-2 $\beta$ -methyl-3 $\alpha$  -vitamin D<sub>3</sub>; (iii) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\alpha$  -methyl-3 $\beta$ -vitamin D<sub>3</sub>; or (iv) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\alpha$  -methyl-3 $\alpha$  -vitamin D<sub>3</sub>. Specification, page 6, lines 13, 16, 17, and 19.

Claim 4 provides a method for producing a vitamin D<sub>3</sub> compound described in claim 3, comprising reacting an exo-methylene compound of formula (II) (as defined in claim 4) with an eneyne compound of formula (III) (as defined in claim 4) in the presence of a palladium catalyst, and optionally removing the protecting group of the tri (C<sub>1</sub> to C<sub>7</sub> hydrocarbon) silyl) group. Specification, page 5, lines 10-30; page 6, lines 1-4; and pages 7-15.

**VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

**1. U.S. Application No. 10/069,481**

Claim 3 was provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3, 7 and 8 of copending U.S. Application No. 10/069,481.

At page 3 of the Office Action of April 20, 2004, the Examiner stated that although the conflicting claims are not identical, they are not patentably distinct from each other because the 2-position of vitamin D represents a lower alkyl group and it is claimed in '481, where vitamin D<sub>3</sub> is a 2-substituted alkyl. The Examiner concluded that in the copending application and the present application the 2-substituted vitamin D<sub>3</sub> compounds which are claimed are considered obvious over each other.

**2. U.S. Patent 5,945,410 and U.S. Patent 6,306,844 B1**

Claim 3 was rejected under 35 U.S.C. §103(a) as being unpatentable over *DeLuca et al.*, U.S. Patent 5,945,410 (US '410) and U.S. Patent 6,306,844 B1 (US '844).

According to the Examiner, both references teach 2-methyl and 2-alkyl 19-nor 20(S) vitamin D<sub>3</sub> compounds. The Examiner further asserted that US '410 teaches 1 $\alpha$ , 25-dihydroxy-2 $\alpha$  and 1 $\alpha$ , 25-dihydroxy-2 $\beta$  methyl 19-nor vitamin D<sub>3</sub> (compound 12 and 13, example 1) and the biological activity of 2-methyl substituted 19-nor 1, 25-(OH)<sub>2</sub>D<sub>3</sub> compounds and their 20-S isomers. The Examiner specifically referred to lines 36-67, in col. 15 and lines 1-37 in col. 16, where 2-methyl substitutions are said to produce surprisingly potent compounds.



**3.      Trost et al, J. Am. Chem. Soc., 114: 9836-45 (1992)**

Claim 4 was rejected under 35 U.S.C. § 103(a) as being unpatentable over Trost et al, J. Am. Chem. Soc., 114: 9836-45 (1992) (Trost).

The Examiner's position was that Trost discloses a palladium-catalyzed alkylative cyclization of enyes for the synthesis of vitamin D derivatives. The Examiner asserted that the difference between the claimed invention and the disclosure of Trost is that in the present invention a different starting material is used wherein the starting compound of the claimed invention has a methyl group at the 4-position of the compound of formula III and Trost discloses an unsubstituted 4-position, but that both are enyes of formula III. Thus, the Examiner concluded that one of ordinary skill in the art would have been motivated to use the process of Trost in order to obtain the instant derivatives since the starting materials would be expected to react similarly.

## **VII. ARGUMENTS**

### **1. The Provisional Obviousness-Type Double Patenting Rejection Of Claim 3 Over U.S. Application No. 10/069,481 Should Be Withdrawn.**

Until cited by the Examiner, Appellants were not aware of the copending application and, furthermore, do not have access to the application or its claims.

Nonetheless, Appellants note that the present application claims benefit to May 2, 1997, whereas the cited application claims benefit to April 28, 1999 (information taken from corresponding international applications).

Accordingly, the obviousness-type double patenting rejection should be removed from the present application and maintained in the cited application, which was the later filed application.

Furthermore, the copending application cited by the Examiner is invalid and unpatentable over Appellants' corresponding International Publication No. WO 98/50353 published November 12, 1998. WO 98/50353 is believed to have been published more than one year prior to the actual U.S. filing date of U.S. Application No. 10/069,481, and therefore is legally effective art under 35 U.S.C. § 102 (b) and/or 103 (a). Accordingly, it is believed that the cited application will not issue.

Therefore, at a minimum, this provisional obviousness-type double patenting rejection should be held in abeyance until a decision on non-patentability is made in the cited application.

### **2. Claim 3 Is Patentable Under 35 U.S.C. § 103(a) Over DeLuca et al., U.S. Patent 5,945,410 (US '410) and U.S. Patent 6,306,844 B1 (US '844).**

The cited US '844 patent is a CIP of U.S. Patent 6,127,559 (US '559).

The US '844 patent has a publication date of October 23, 2001, and a filing date of July 14, 2000, both of which are after Appellants' actual U.S. filing date of December 29, 1998. Similarly, the US '559 patent has a publication date of October 3, 2000, and a filing date of August 17, 1998, both of which are after Appellants' April 30, 1998, PCT filing date.

Therefore, the Examiner cannot rely upon any disclosure that is not also in the US '410 patent. In order to overcome this rejection, Appellants submitted two declarations under 37 C.F.R. § 1.131, establishing that the present inventors actually reduced to practice at least two species of the invention prior to the March 17, 1997 filing date of US '410. Therefore, the *DeLuca* references should be removed and the rejection withdrawn.

More specifically, an executed Rule 131 Declaration by Dr. Toshie Fujishima, one of the inventors, was submitted on December 18, 2002. Also submitted therewith as corroboration was an executed Rule 131 Declaration by Dr. Zhaopeng Liu.

In her declaration, Dr. Fujishima explains that prior to March 17, 1997, she synthesized compound (68), (20S)-1 $\alpha$ ,25-dihydroxy-2 $\beta$ -methyl-3 $\beta$ -vitamin D<sub>3</sub> (Example 2, page 33 of the specification) and compound (72), (20S)-1 $\alpha$ ,25-dihydroxy-2 $\alpha$ -methyl-3 $\beta$ -vitamin D<sub>3</sub> (Example 1, page 32 of the specification). The synthesis of compound (68) is documented in experimental note (1), Exhibit 1, and the synthesis of compound (72) is documented in experimental note (2), Exhibit 1, both of which are copies of pages from Dr. Fujishima's notebook. The entries on the pages are described in detail in the declaration. Dr. Fujishima also describes NMR spectra of compound (68) (Chart 1, Exhibit 1) and of compound (72) (Chart 2, Exhibit 1) from an analysis that was carried out prior to March 17, 1997. Dr. Fujishima testifies that she reviewed the NMR spectra prior to March 17, 1997 and identified the compounds as those compounds designated as compound (68) and compound

(72) in the present application. In addition Dr. Fujishima testifies that the vitamin D receptor affinity of compound (68) and of compound (72) was measured prior to March 17, 1997 (note 3 and Chart 3, Exhibit 1). Further, Dr. Fujishima testifies that she presented the results of the work to her colleagues prior to March 17, 1997. Handouts from the seminar were submitted with the declaration, and are explained in detail by Dr. Fujishima (Exhibit 2).

The declaration of Dr. Liu corroborates Dr. Fujishima's testimony. Specifically, Dr. Liu states that he remembers that Dr. Fujishima is the author of experimental notes (1), (2), and (3) (Exhibit 1) and that he recalls that Dr. Fujishima planned to present the results of these notes at a workshop. Dr. Liu testifies that to do so, Dr. Fujishima had to submit an abstract, which was due prior to March 17, 1997 (Exhibit 3). Dr. Liu also states that he was present when Dr. Fujishima presented the results of her work on compounds (68) and (72) in a group seminar held prior to March 17, 1997 (Exhibit 2). In his declaration, Dr. Liu describes in detail the handouts from the seminar and states that he understood that Dr. Fujishima had synthesized and confirmed the usefulness of compounds (68) and (72) prior to March 17, 1997. Dr. Liu also states that he understood that Dr. Fujishima had determined a detailed scheme for how to make compounds (68) and (72) prior to March 17, 1997.

As evidenced by the Fujishima and Liu declarations, two species within the claimed invention had been actually reduced to practice prior to the March 17, 1997 filing date of US '410. Accordingly, Appellants have antedated US '410.

For at least the reasons set forth above, claim 3 is patentable over US '410 and US '844.

**3. Claim 4 Is Patentable Under 35 U.S.C. § 103(a) Over Trost M. Barry et al., J. Am. Chem. Soc., 114: 9836-45 (1992) (Trost).**

The Examiner has not made a *prima facie* showing of obviousness. To establish a *prima facie* case of obviousness there must be (1) some suggestion or motivation within the reference or in the knowledge generally available to one of ordinary skill in the art to modify the reference; (2) a reasonable expectation of success; and (3) the reference relied upon by the Examiner must teach or suggest all of the claimed limitations. See *Hodesh v. Block Drug Co*, 786 F.2d 1136, 1153, n.5, 229 USPQ 182, 187, n.5 (Fed. Cir. 1986); *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 1438 (Fed. Cir. 1991); and *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974).

In this case, there is no teaching or suggestion within the reference to modify the disclosed process such that one of ordinary skill in the art would have had a reasonable expectation of success in achieving the claimed 20(S) vitamin D<sub>3</sub> derivatives, having unexpectedly superior properties as shown in the specification on page 39 and in the Declarations under 37 C.F.R. § 1.132 submitted December 30, 1999, (the executed declaration was filed on August 1, 2000) and on November 13, 2001. It has been established that where a method for preparing purified isomers of compounds, such as the claimed 20(S) vitamin D<sub>3</sub> derivatives, is not taught or suggested by a reference, a *prima facie* case of obviousness has not been established. Appellants rely on the holding of *Emory University v. Glaxo Wellcome, Inc.* (44 USPQ2d 1407 (DC NGa 1997)) citing *In re Hoeksema* (158 USPQ 596, (CCPA 1968)) for the holding that "if the prior art of record fails to disclose or render obvious a method for making a claimed compound at the time the invention was made, it may not be legally concluded that the compound itself is in the possession of the public."

In addition, the Examiner failed to consider the claimed invention as a whole. The reference does not teach all of the limitations of the claimed invention, namely, the specifically recited 20(S) form vitamin D<sub>3</sub> derivative compounds. The fact that the reference may teach a similar process is not sufficient by itself to establish obviousness under the fact intensive inquiry required by 35 U.S.C. § 103. *See In re Ochiai*, 71 F.3d 1565, 37 USPQ2d 1127 (Fed. Cir. 1995) (reversing the Board of Appeals and the Examiner stating that both had used incorrect methodology in determining obviousness of a process for making a compound based upon a “general obviousness rule that a claim is obvious if prior art references disclose the same general process using similar starting materials” and stating that no such *per se* rule exists).

As in the case of *In re Ochiai*, the Examiner’s rejection is based upon a general rule of obviousness that a process claim is obvious if the prior art references disclose the same general process using similar starting materials. The Examiner has maintained the position that the starting materials of the claimed invention are analogous to those taught by *Trost et al* because they are both enynes of formula III. As a basis for the rejection the Examiner stated and maintained: “[i]t has been held that application of an old process to a[n] analogous material to obtain a result consistent with the teachings of the art would have been obvious to one having ordinary skill.” However such an analysis does not take into consideration the fact that the compounds of formula III of the claimed invention are different as well as the fact that the recited 20(S) vitamin D<sub>3</sub> derivative compounds are different and have been shown to possess unexpectedly superior properties over the closest specifically disclosed compounds.

Further, the compound of claim 4 is an intermediate compound rather than a starting material, as incorrectly stated by the Examiner, and the intermediate compound is essential for the claimed process with respect to the preparation of the specific 20(S) form vitamin D recited in the claims. The data showing unexpectedly superior results of the final product is sufficient to establish the patentability of the claimed process.

For at least the reasons set forth above, claim 4 is patentable over Trost.

**4. The Claims Do Not Stand And Fall Together**

Claims 3 and 4 are directed to a compound and a method of making that compound. The claims are separately patentable (*see In re Ochiai*, 71 F.3d 1565).

APPEAL BRIEF UNDER 37 C.F.R. § 41.37  
U.S. Appln. No.: 09/214,155

Unless a check is submitted herewith for the fee required under 37 C.F.R. §41.37 and 1.17(c), please charge said fee to Deposit Account No. 19-4880.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



Susan J. Mack  
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WASHINGTON OFFICE

**23373**

CUSTOMER NUMBER

Date: October 20, 2004



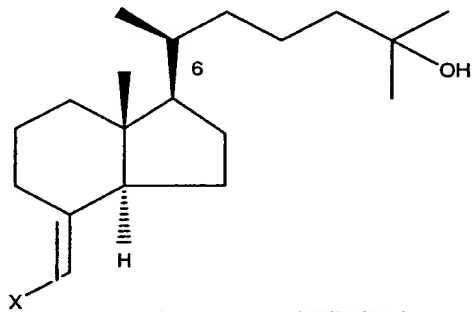


**CLAIMS ON APPEAL**

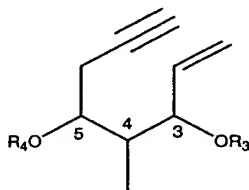
**Claim 3.** A 1,25-dihydroxy-2-methylvitamin D<sub>3</sub> compound, wherein the compound is

- (i) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\beta$ -methyl-3 $\beta$ -vitamin D<sub>3</sub>;
- (ii) (20S)-1  $\beta$ , 25-dihydroxy-2 $\beta$ -methyl-3 $\alpha$  -vitamin D<sub>3</sub>;
- (iii) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\alpha$  -methyl-3 $\beta$ -vitamin D<sub>3</sub>; or
- (iv) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\alpha$  -methyl-3 $\alpha$  -vitamin D<sub>3</sub>.

**Claim 4.** A method for producing a vitamin D<sub>3</sub> compound described in claim 3, comprising reacting an exo-methylene compound of formula (II):



wherein X is a bromine atom or an iodine atom, with an eneyne compound of formula (III):



wherein R<sub>3</sub> and R<sub>4</sub> are each independently a hydrogen atom or a tri (C<sub>1</sub> to C<sub>7</sub> hydrocarbon) silyl) group in the presence of a palladium catalyst, and optionally removing the

protecting group of the tri (C<sub>1</sub> to C<sub>7</sub> hydrocarbon) silyl) group, and further wherein the vitamin D<sub>3</sub> compound is

- (i) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\beta$ -methyl-3 $\beta$ -vitamin D<sub>3</sub>;
- (ii) (20S)-1 $\beta$ , 25-dihydroxy-2 $\beta$ -methyl-3 $\alpha$ -vitamin D<sub>3</sub>;
- (iii) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\alpha$ -methyl-3 $\beta$ -vitamin D<sub>3</sub>; or
- (iv) (20S)-1 $\alpha$ , 25-dihydroxy-2 $\alpha$ -methyl-3 $\alpha$ -vitamin D<sub>3</sub>.

## EVIDENCE APPENDIX

Pursuant to 37 C.F.R. § 41.37(ix), submitted herewith are copies of any evidence submitted pursuant to 37 C.F.R. §§ 1.130, 1.131, or 1.132 or any other evidence entered by the Examiner and relied upon by Appellant in the appeal.

1. Executed Rule 131 Declaration by Dr. Toshie Fujushima, submitted December 18, 2002.
2. Executed Rule 131 Declaration by Dr. Zhaopeng Liu, also submitted December 18, 2002.
3. Executed Rule 132 Declaration by Dr. Ishizuka, submitted August 1, 2000.
4. Executed Rule 132 Declaration by Dr. Miura, submitted November 13, 2001.
5. Exhibit 1, submitted on December 18, 2002 with the executed Declarations of Drs. Toshie Fujushima and Zhaopeng Liu.
6. Exhibit 2, submitted on December 18, 2002 with the executed Declarations of Drs. Toshie Fujushima and Zhaopeng Liu.
7. Exhibit 3, submitted on December 18, 2002 with the executed Declarations of Drs. Toshie Fujushima and Zhaopeng Liu.

## RELATED PROCEEDINGS APPENDIX

Submitted herewith are copies of decisions rendered by a court or the Board in any proceeding identified about in Section II pursuant to 37 C.F.R. § 41.37(c)(1)(ii).

NONE



**PATENT APPLICATION**

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In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155

Group Art Unit: 1616

Filed: December 29, 1998

Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

**DECLARATION UNDER 37 C.F.R. § 1.132**

Commissioner for Patents  
Washington, D.C. 20231

**FILED**  
**NOV 13 2001**

Sir:

I, Daishiro Miura, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1990 and a Ph.D. degree in 1999 from Hokkaido University;

THAT I am a member of several Japanese Scientific Societies related to research in toxicology, hematology, vitamin D and bone and mineral metabolism.

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1990, in the Safety Research Department, where I have been involved in the toxicity study of pharmaceuticals and biological activities of Vitamin D<sub>3</sub>.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the final Office Action of June 12, 2001 issued in reference to the application. In response to the final Office Action, I submit herewith this Declaration comparing the potency of 20(S)-forms versus 20(R)-forms of Vitamin D derivatives and as evidence that the 20(S)-forms are significantly more potent than the 20(R)-forms in their ability to induce cellular differentiation.

**Comparison of Activity for 20(S)- versus 20(R)-forms of Vitamin D derivatives for induction of HL-60 cell differentiation.**

**Materials and methods.**

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passed by culturing. Cells which had been treated by successive culturing for one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 ( $1-2 \times 10^5$  cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum (FBS). Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of  $2 \times 10^4$  cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well.

An ethanol solution containing the 20(S)-form compounds, (68), (71), (72) or (74), at concentrations ranging from  $1 \times 10^{-9}$  M to  $1 \times 10^{-6}$  M (for Table 1) or  $1 \times 10^{-7}$  M to  $1 \times 10^{-4}$  M (for Table 2) was added to each well at 1  $\mu$ l/well. Further, regarding  $1\alpha,25-(\text{OH})_2\text{D}_3$ , an ethanol solution containing  $1 \times 10^{-7}$  M to  $1 \times 10^{-4}$  M of the compound was added at 1  $\mu$ l/well, and for the control, ethanol alone was added at 1  $\mu$ l/well.

An ethanol solution containing the 20(R)-form compounds, (3), (4), (6) or (65), at concentrations ranging from  $1 \times 10^{-7}$  M to  $1 \times 10^{-4}$  M was added to each well at 1  $\mu$ l/well. For the control, ethanol alone was added at 1  $\mu$ l/well.

After culturing at 37°C for 4 days under a 5% CO<sub>2</sub> atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reducing activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min. Then, cells were collected by centrifugation, resuspended in FBS and cytospin smears were prepared. After air-drying, smears were stained with Kernschrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reducing) was determined under an optical microscope.

#### **Results.**

The results are shown in the following Table 1 (Comparison of activity for 20(S)-form compounds at concentrations ranging from  $1 \times 10^{-12}$  M to  $1 \times 10^{-9}$  M and 20(R)-form compounds at concentrations ranging from  $1 \times 10^{-10}$  M to  $1 \times 10^{-7}$  M) and Table 2 (Activity for 20(S)-form compounds at concentrations ranging from  $1 \times 10^{-10}$  M to  $1 \times 10^{-7}$  M). These results shown in Table 1 and Table 2 were obtained from independent two experiments.

**Table 1.**

Sample	Concentration (M)	% Cells showing NBT activity	Sample	Concentration (M)	% Cells showing NBT activity
Control		1.5	Control		5.8
1 $\alpha$ ,25-(OH) $_2$ D $_3$	10 $^{-10}$	4.3 $\pm$ 1.2			
	10 $^{-9}$	36.8 $\pm$ 2.0			
	10 $^{-8}$	86.1 $\pm$ 2.6			
	10 $^{-7}$	96.5 $\pm$ 1.0			
<b>20(S)-form</b>			<b>20(R)-form</b>		
Compound (68) <sup>1</sup>	10 $^{-12}$	1.7 $\pm$ 0.3	Compound (65) <sup>2</sup>	10 $^{-12}$	—
	10 $^{-11}$	2.8 $\pm$ 0.7		10 $^{-11}$	—
	10 $^{-10}$	57.7 $\pm$ 5.0		10 $^{-10}$	1.8 $\pm$ 0.7
	10 $^{-9}$	95.7 $\pm$ 1.0		10 $^{-9}$	1.4 $\pm$ 0.8
	10 $^{-8}$	—		10 $^{-8}$	32.0 $\pm$ 3.2
	10 $^{-7}$	—		10 $^{-7}$	95.1 $\pm$ 2.8
Compound (71) <sup>3</sup>	10 $^{-12}$	1.5 $\pm$ 0.8	Compound (3) <sup>4</sup>	10 $^{-12}$	—
	10 $^{-11}$	1.8 $\pm$ 0.8		10 $^{-11}$	—
	10 $^{-10}$	2.0 $\pm$ 1.0		10 $^{-10}$	1.6 $\pm$ 1.1
	10 $^{-9}$	40.5 $\pm$ 1.8		10 $^{-9}$	1.3 $\pm$ 0.5
	10 $^{-8}$	—		10 $^{-8}$	1.8 $\pm$ 0.7
	10 $^{-7}$	—		10 $^{-7}$	1.4 $\pm$ 0.2
Compound (74) <sup>5</sup>	10 $^{-12}$	6.4 $\pm$ 1.1	Compound (6) <sup>6</sup>	10 $^{-12}$	—
	10 $^{-11}$	17.0 $\pm$ 2.3		10 $^{-11}$	—
	10 $^{-10}$	16.7 $\pm$ 1.1		10 $^{-10}$	1.9 $\pm$ 0.6
	10 $^{-9}$	96.4 $\pm$ 1.4		10 $^{-9}$	1.8 $\pm$ 0.2
	10 $^{-8}$	—		10 $^{-8}$	12.9 $\pm$ 2.2
	10 $^{-7}$	—		10 $^{-7}$	92.7 $\pm$ 2.9
Compound (72) <sup>7</sup>	10 $^{-12}$	3.7 $\pm$ 0.8	Compound (4) <sup>8</sup>	10 $^{-12}$	—
	10 $^{-11}$	94.4 $\pm$ 1.8		10 $^{-11}$	—
	10 $^{-10}$	95.7 $\pm$ 2.3		10 $^{-10}$	2.3 $\pm$ 0.9
	10 $^{-9}$	96.2 $\pm$ 2.0		10 $^{-9}$	69.4 $\pm$ 3.9
	10 $^{-8}$	—		10 $^{-8}$	93.9 $\pm$ 4.4
	10 $^{-7}$	—		10 $^{-7}$	95.9 $\pm$ 1.1

<sup>1</sup> Example 2, page 33 of the specification.

<sup>2</sup> Page 40, line 1 of the specification.

<sup>3</sup> Example 5, page 36 of the specification.

<sup>4</sup> Page 39, line 30 of the specification.

<sup>5</sup> Example 7, page 37 of the specification.

<sup>6</sup> Page 39, line 33 of the specification.

<sup>7</sup> Example 1, page 32 of the specification.

<sup>8</sup> Page 39, line 31 of the specification.



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**Table 2.**

Sample	Concentration (M)	% Cells showing NBT activity
Compound (68)	$10^{-10}$	90.8
	$10^{-9}$	98.3
	$10^{-8}$	98.0
	$10^{-7}$	99.5
Compound (71)	$10^{-10}$	22.0
	$10^{-9}$	47.1
	$10^{-8}$	97.5
	$10^{-7}$	97.7
Compound (74)	$10^{-10}$	52.1
	$10^{-9}$	95.4
	$10^{-8}$	97.7
	$10^{-7}$	98.7
Compound (72)	$10^{-10}$	93.7
	$10^{-9}$	97.9
	$10^{-8}$	98.6
	$10^{-7}$	98.6

**Conclusions.**

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20(S)-forms show excellent efficacy compared to the 20(R)-forms. Comparison of compound (68) with compound (65); compound (71) with compound (3); compound (74) with compound (6) and compound (72) with compound (4) reveals that the 20(S)-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: November 7, 2001

Daishiro Miura  
Daishiro Miura



Yours:

12/16

3m: 7-335

PATENT APPLICATION  
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155

Group Art Unit: 1616

Filed: December 29, <sup>1998</sup>~~1999~~  
*Seiichi Ishizuka*

Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents  
Washington, D.C. 20231

FILED

AUG - 1 2000

Sir:

I, Seiichi Ishizuka, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1974 and a Ph.D. degree in 1996 from Tohoku University;

THAT I am a member of several Japanese and American Scientific Societies related to research in vitamin D and bone and mineral metabolism, a referee for two scientific journals (Biochemistry and J. Nutritional Biochemistry), and the recipient of two scientific research awards (Vitamin D research award from Brown University (1995) and scientific research award from Japanese Society for Bone and Mineral Research (1997)).

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1975, in the Department of Bone and Calcium Metabolism, where I have been involved in the study of the metabolism, biological activities and mechanism of actions of Vitamin D<sub>3</sub>.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the Office Action of June 30, 1999 issued in reference to the application. In response to the Office Action, I have conducted the following experiments to investigate the activity of 20R-forms for the vitamin D<sub>3</sub> derivatives disclosed in the reference cited by the Examiner for their effect on nitroblue tetrazolium reduction activity in the HL-60 cell line compared to the instant 20S-forms for the derivatives. A description of the experiments and the results obtained are the following.

**Inventive Example 1 (20S-form of vitamin D<sub>3</sub> derivative)**

Activity for 20S-forms of Vitamin D derivatives on induction of HL-60 cells to differentiate.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passaged by culturing. Cells which had been treated by successive culturing for about one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10<sup>5</sup> cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10<sup>4</sup> cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well. An ethanol solution containing compounds 68, 71, 72 or 74 at

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concentrations ranging from  $1 \times 10^{-9}$  M to  $1 \times 10^{-6}$  M was added to each well at 1  $\mu$ l/well. Further, regarding  $1 \alpha, 25(\text{OH})_2\text{D}_3$ , an ethanol solution containing  $1 \times 10^{-7}$  M to  $1 \times 10^{-4}$  M of the compound was added at 1  $\mu$ l/well, and for the control, ethanol alone was added at 1  $\mu$ l/well. After culturing at 37°C for 4 days under a 5%  $\text{CO}_2$  atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for a cytospin centrifugation. After air drying, the cell pellet was stained with Kernschrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope. The results are shown in the following Table.

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Nitroblue Tetrazolium Reduction Activity in HL-60 Cells Treated with 20S-forms of Vitamin D  
Derivatives

Compound	Concentration (M)	% cells showing nitroblue tetrazolium reduction activity
Control		1.5
1 $\alpha$ ,25-(OH) <sub>2</sub> D <sub>3</sub>	10 <sup>-10</sup>	4.3±1.2
	10 <sup>-9</sup>	36.8±2.0
	10 <sup>-8</sup>	86.1±2.6
	10 <sup>-7</sup>	96.5±1.0
Compound (68)	10 <sup>-12</sup>	1.7±0.3
	10 <sup>-11</sup>	2.8±0.7
	10 <sup>-10</sup>	57.7±5.0
	10 <sup>-9</sup>	95.7±1.0
Compound (71)	10 <sup>-12</sup>	1.5±0.8
	10 <sup>-11</sup>	1.8±0.8
	10 <sup>-10</sup>	2.0±1.0
	10 <sup>-9</sup>	40.5±1.8
Compound (74)	10 <sup>-12</sup>	6.4±1.1
	10 <sup>-11</sup>	17.0±2.3
	10 <sup>-10</sup>	16.7±1.1
	10 <sup>-9</sup>	96.4±1.4
Compound (72)	10 <sup>-12</sup>	3.7±0.8
	10 <sup>-11</sup>	94.4±1.8
	10 <sup>-10</sup>	95.7±2.3
	10 <sup>-9</sup>	96.2±2.0

### Comparative Example 1 (20R form of vitamin D<sub>3</sub> derivatives)

Activity for 20R-forms of Vitamin D derivatives on induction of HL-60 cells to differentiate.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passaged by culturing. Cells which had been treated by successive culturing for about one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10<sup>5</sup> cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10<sup>4</sup> cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well. An ethanol solution containing compounds 3, 4, 6 or 65 at concentrations ranging from 1x10<sup>-7</sup> M to 1x10<sup>-3</sup> M was added to each well at 1 µl/well. For the control, ethanol alone was added at 1 µl/well. After culturing at 37°C for 4 days under a 5% CO<sub>2</sub> atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in fresh culture medium to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for cytospin centrifugation. After air drying, the cell pellet was stained with Kernschrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope. The results are shown in the following Table.

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Nitroblue Tetrazolium Reduction Activity in HL-60 Cells Treated with 20R-forms of Vitamin D  
Derivatives

Compound	Concentration (M)	% cells showing nitroblue tetrazolium reduction activity
Control		5.8
Compound (65)	$10^{-8}$	6.8
	$10^{-7}$	65.8
	$10^{-6}$	88.3
Compound (3)	$10^{-8}$	6.8
	$10^{-7}$	11.4
	$3 \times 10^{-7}$	80.9
Compound (6)	$10^{-9}$	5.2
	$10^{-8}$	17.0
	$10^{-7}$	71.2
	$10^{-6}$	82.5
Compound (4)	$10^{-10}$	8.1
	$10^{-9}$	27.9
	$10^{-8}$	80.0
	$10^{-7}$	88.7
	$10^{-6}$	94.5

Conclusions.

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20S-forms show excellent efficacy compared to the 20R-forms. Comparison of compound (4) with compound (72); compound (65) with compound (68); compound (6) with compound (74) and compound (3) with compound (71) reveals that the 20S-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.



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I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 4, 2000

Seiichi Ishizuka  
Seiichi Ishizuka



28007

**PATENT APPLICATION  
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155

Group Art Unit: 1616

Filed: December 29, 1998

Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

**FUJISHIMA DECLARATION UNDER 37 C.F.R. § 1.131**

Commissioner for Patents  
Washington, D.C. 20231

Sir:

**FILED**

DEC 18 2002

I, Toshie Fujishima, hereby declare and state:

THAT I am a citizen of Japan;

THAT I received a Masters Degree in 1993 and a Ph.D. degree in 1996 from Tokyo University;

THAT I am a member of several Japanese Scientific Societies related to research in organic chemistry and Vitamin D;

THAT I have been employed by Teikyo University, Faculty of Pharmaceutical Sciences since 1996, where I have been involved in the synthetic study of Vitamin D<sub>3</sub> in Professor TAKAYAMA's group.

I am one of the joint inventors of the invention claimed in the above-identified application.

I have a thorough knowledge of the invention in the above-identified patent application, and I have read the non-final Office Action of June 10, 2002 issued in reference to the application. In response to the non-final Office Action, I submit herewith this Declaration, which explains that I am the author of the experimental note of certain compounds claimed in the application and which explains my role in inventing the subject matter of the claims of the application.

**FUJISHIMA DECLARATION UNDER 37 C.F.R. § 1.131**  
**U.S. Appl. No. 09/214,155**

**Author of experimental notes**

I am the author of experimental notes (1), (2), and (3). (*see* Exhibit 1).

**My role in the invention claimed in the application**

I synthesized compound (68) (Example 2, page 33 of the specification) prior to March 17, 1997, as described in experimental note (1) and compound (72) (Example 1, page 32 of the specification) prior to March 17, 1997, as described in experimental note (2). I ordered a NMR analysis of compound (68) and compound (72) from Instrument Analysis Center of Teikyo University, Faculty of Pharmaceutical Sciences, and the analysis was carried out prior to March 17, 1997 by Ms. Junko Shimode, a NMR operator at the center. Corresponding NMR spectra are shown in Exhibit 1, Chart 1 (Compound (68)) and Chart 2 (Compound (72)). I reviewed the NMR spectra prior to March 17, 1997, and I identified the compounds as those compounds designated compound (68) and compound (72) in the present application. I measured the Vitamin D receptor (VDR) affinity of compound (68) and compound (72) prior to March 17, 1997, as described in experimental note (3). Corresponding data from a liquid scintillation counter is shown in Exhibit 1, Chart 3. I presented the results of the work to my colleagues in one of Professor TAKAYAMA's group seminars prior to March 17, 1997. (*see* Exhibit 2)

Prior to March 17, 1997, I also submitted an abstract for a poster session at the Tenth Workshop on Vitamin D. (*see* Exhibit 3)

These activities are described in detail below with reference to the notebook pages and actual data. Because the text in my notebook and on the data is in Japanese, I have also included copies of the pages and data with English text added.

Exhibit 1 is a compilation of data and pages from my notebook number 2, which I kept in the ordinary course of my research. The entries are in my handwriting.

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Note 1 is dated in the upper left-hand corner. The date is prior to March 17, 1997. This page shows synthesis of compound #346 from the protected precursor compound #345. Compound #346 is designated compound (68) in the above-identified application.

The upper left corner of the page also has the notation "#346." This means that I planned to make compound #346. The structure of synthesized compound #346 is shown in the upper right of the page. The structure is the same as that for the compound designated compound (68) in the specification of the above-identified application. The compound shown in the upper left of the page is the protected precursor. "TBS" stands for tert-butyldimethylsilyl. The arrow pointing from protected precursor compound #345 to synthesized compound #346 indicates that I planned to make synthesized compound #346 from protected precursor compound #345.

Below the structural formulas in a bracket is the notation "#345 work up," which means that I used compound #345 as a starting compound. I dissolved compound #345 in 1 ml of methanol (MeOH) and then added 11mg of CSA (Camphor sulfonic acid). The notations "20:30~" and "11:00," which appear to the right side of the open bracket mean that the reaction mixture was stirred from 20:30 (eight thirty PM), to 11:00 (eleven o'clock). The dates, which have been blocked out, indicate that the stirring time was about 38 ½ hours.

Below the open bracket, I indicate that the mixture was stirred at room temperature (rt) and then the methanol was evaporated from the reaction mixture. To the residue I added water and then extracted with EA (ethyl acetate). The extract was washed with brine, dried over MgSO<sub>4</sub>, filtered, and evaporated to give compound #346.

Below this description I indicate that the crude product was purified by silica gel chromatography and further by HPLC (ODS (18)). I then ordered a <sup>1</sup>H NMR analysis of the compound.

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Chart 1 is dated prior to March 17, 1997. Chart 1 is a  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound #346 i.e. compound (68) and its numerically outputted data. This data was assigned as compound #346 as follows:  $\delta$  0.55 (3 H, s), 0.85 (3 H, d,  $J = 6.4$  Hz), 1.15 (3 H, d,  $J = 6.7$  Hz), 1.21 (6 H, s), 2.42 (1 H, dd,  $J = 13.9, 4.9$  Hz), 2.52 (1 H, br. d,  $J = 13.9$  Hz), 2.82 (1 H, dd,  $J = 11.9, 4.0$  Hz), 4.02 (2 H, m), 5.02 (1 H, t,  $J = 1.8$  Hz), 5.37 (1 H, t,  $J = 1.8$  Hz), 6.03 (1 H, d,  $J = 11.3$  Hz), 6.35 (1 H, d,  $J = 11.3$  Hz).

Note 2 is dated in the upper left-hand corner. The date is prior to March 17, 1997. This page shows synthesis of compound #344 from the protected precursor compound #343. Compound #344 is designated compound (72) in the above-identified application.

The upper left corner of the page also has the notation "#344." This means that I planned to make compound #344. The structure of compound #344 is shown in the upper right of the page. The structure is the same as that for the compound designated compound (72) in the specification of the above-identified application. The compound shown in the upper left of the page is the protected precursor. "TBS" stands for tert-butyldimethylsilyl. The arrow pointing from compound #344 to compound #343 indicates that I planned to make compound #344 from compound #343.

In a bracket below the structural formulas is the notation "#343 work up," which means that I used compound #343 as a starting compound. I dissolved compound #343 in 1 ml of methanol (MeOH) and then added 11 mg of CSA (camphor sulfonic acid).

Below the open bracket, I indicate that the mixture was stirred from 14:20 (two twenty PM) to 9:00 (nine o'clock), the next day under Ar gas at room temperature (rt), and then the methanol was distilled away from the reaction mixture. To the residue I added water and then extracted with EA (ethyl acetate). The extract was washed with brine, dried over  $\text{MgSO}_4$ , dehydrated, filtered, and evaporated to give compound #344.

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Below this description I indicate that the crude product was purified by silica gel chromatography and further by HPLC (ODS (18)). " $\phi$  0.9 cm 10 cm height" means silica gel column size (0.9 cm diameter and 10 cm length). "EA : n-hex = 1:1" indicates the solvent system for the chromatography (ethyl acetate : n-hexane = 1 : 1).

Drawings at the bottom of the page are illustrations of TLC (thin layer chromatography). "EA : n-hex = 1:1" means a solvent system of the TLC (ethyl acetate : n-hexane = 1 : 1). "SM" and "RM" mean starting material and reaction mixture. Circles in the illustrations show spots on TLC. TLC is a typical monitor system of organic reaction because the spots show compound(s) in analyzed material. A position difference between the spot labeled "SM" and the spot labeled "RM" means that the compound(s) in the "SM" and "RM" spots were different and the reaction was complete.

I then ordered a  $^1\text{H}$  NMR analysis of the compound.

Chart 2 is dated prior to March 17, 1997. Chart 2 is a  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of compound #344 i.e. compound (72) and its numerically outputted data. This data assigned as compound #344 as follows:  $\delta$  0.53 (3 H, s), 0.85 (3 H, d,  $J = 6.7$  Hz), 1.08 (3 H, d,  $J = 6.8$  Hz), 1.21 (6 H, s), 2.23 (1 H, dd,  $J = 13.4, 7.9$  Hz), 2.67 (1 H, dd,  $J = 13.4, 4.0$  Hz), 2.83 (1 H, m), 3.83 (1 H, ddd,  $J = 7.9, 4.4, 4.0$  Hz), 4.29 (1 H, d,  $J = 3.3$  Hz), 5.01 (1 H, d,  $J = 1.8$  Hz), 5.28 (1 H, m), 6.01 (1 H, d,  $J = 11.3$  Hz), 6.39 (1 H, d,  $J = 11.3$  Hz).

Note 3 is dated prior to March 17, 1997. The 1st through 3rd pages show the employed protocol for VDR binding assay.

The 1st page shows the 1st to the 8th steps of the protocol. The 1st, I made phosphate buffer and kept it at 4 °C. The 2nd, I prepared diluted solutions of  $1\alpha,25(\text{OH})_2\text{VD}_3$ , compound #344 and compound #346. The 3rd, I prepared  $[26,27\text{-methyl}^3\text{H}]1\alpha,25(\text{OH})_2\text{VD}_3$  solution, took 100  $\mu\text{l}$  of the solution and evaporated, then I added 6.25 ml of Japanese pharmacopeia grade ethanol to the solution. The 4th, I

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poured 50 µl of the sample in Japanese pharmacopeia grade ethanol (made in the 2nd step) into disposable culture tubes (12 x 75 mm, product of IWAKI) in order of concentration from thin to dense by dispenser (for example from tube number 14, 28 to 1, 15). Into tubes from number 85 to 96 was poured Japanese pharmacopeia grade ethanol only. The 5th, I made a receptor solution (lot 110431, product of YAMASA). I poured 5 ml of phosphate-potassium buffer (made in the 1st step) into a vessel containing the thymus receptor, dissolved the receptor gently, added further 50 ml of the buffer and stirred gently. The 6th, I added 500 µl of the receptor solution to each tube except blank tubes (tube number 89, 90, 91, 92), and I added 500 µl of the buffer solution to blank tubes. The 7th, I stirred solutions in the tubes (made in the 6th step) by vortex without foaming. The 8th, I pre-incubated the solutions in the tubes at room temperature (approximately 22 °C) for 1 hour (13:40 - 14:40). The tops of the tubes were sealed with plastic wrap and aluminum foil.

The 2nd page shows the 9th to the 11th steps of the protocol. These steps were carried out in RI (radio isotope) room. The 9th, I added 50 µl of the radioactive solution (made in the 3rd step) to each tube by dispenser. In the case of radioactivity only count (tube number 97, 98, 99, 100), radioactive solution was added to the vial. The 10th, I stirred the solution in the tubes by vortex without foaming. The 11th, I sealed the top of the tubes with plastic wrap, put the tubes in a 4 °C refrigerator in the RI room, and stood the tubes overnight from 15:10 (ten after three PM). Then I added 10 ml of ACS-II (Aqueous counting scintillant, product of Amersham) to the tubes and measured radioactivity count for 1 min by Aloka A machine (Scintillation counter). Then I stood the tubes at rt, and the next day I measured the radioactivity count for 2 min with other samples. The data measured for 1 min is shown at the middle of this page. "97 16217.7 dpm" means radioactivity count of tube number 97 was 16217.7 disintegrations per minute. "Average 16370 dpm" means calculated average value of tubes 97, 98, 99, 100 counts which are radioactive only solution. "Average 45 dpm" means calculated average value of tubes 101, 102, 103, 104

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counts which are blank solution. In the parenthesis at the bottom of this page there is a calculation of the [26,27-methyl<sup>3</sup>H]1 $\alpha$ ,25(OH)<sub>2</sub>VD<sub>3</sub> weight per tube. 16370 dpm is  $16370/60 = 273$  dps (disintegrations per second), 273 dps is 273 Bq (dps unit equals Bq - becquerel- unit). The radioactivity of the [26,27-methyl<sup>3</sup>H]1 $\alpha$ ,25(OH)<sub>2</sub>VD<sub>3</sub> solution used in the 3rd step was 11.4 GBq/mg, (= 11.4 Bq/pg), so the amount of [26,27-methyl<sup>3</sup>H]1 $\alpha$ ,25(OH)<sub>2</sub>VD<sub>3</sub> was  $273/11.4 = 24$  pg / tube.

The 3rd page shows from the 12th to the 17th steps of the protocol. I carried out these steps the day after the previous steps were done. The 12th, I took the previous day's samples from the refrigerator in the RI room and added 200  $\mu$ l of DCC (dextran coated charcoal) solution (lot M602 product of YAMASA) to each tube by dispenser except total count tubes (tube number 93, 94, 95, 96). And I added 200  $\mu$ l of buffer (made in the 1st step) to each of the total count tubes. The 13th, I stirred the tubes by vortex. The 14th, I stood the tubes for 30 min (9:50 - 10:20) at 4 °C. The 15th, the tubes were centrifuged at 3000 rpm for 10 min (10:30 - 10:40) at 0 °C. The 16th, I transferred 500  $\mu$ l of supernatant of each tube to 20 ml WERATON vial in concentration order from thin to dense (for example from tube number 1 to 14 by same pipetter tip, changed pipetter tip, then from tube number 15 to 28). At that time the vials were put on tray with ice. The 17th, I added 9.5 ml of ACS-II to each of the tubes, shook them and measured its radioactivity count (2min) by Aloka A machine.

The 4th page shows a graph of the radioactivity count (Y-axis) of compounds #346, #344 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD<sub>3</sub> (control) solutions at various concentration (X-axis) at the 11th step of the assay protocol. Closed circles (VD) and closed triangle (VD-2) show 1 $\alpha$ ,25(OH)<sub>2</sub>VD<sub>3</sub> values, open circles (344) and crosses (344-2) show #344 compound values and closed diamonds (346) show #346 compound values. These data were measured by Aloka C machine for 1 min. I measured the data from tube number 1 to 70.

The 5th page shows a table of the radioactivity count (dpm) of the compounds #346, #344 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD<sub>3</sub> (control) solutions at various concentrations. The first column shows the compound's



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concentration, the 2nd and 3rd columns show the radioactivity counts of  $1\alpha,25(\text{OH})_2\text{VD}_3$  (duplicate experiment), the 4th and 5th columns show the radioactivity counts of #344 compound (duplicate experiment) and the 6th and 7th columns show the radioactivity counts of #346 compound (duplicate experiment). The small numerals on the line show tube number.

The 6th page shows a table of the radioactivity count of control solutions. "0" in the 1st column means the concentration of the compound is 0, "blank" right below "0" means buffer solution (made in the 1st step of the assay protocol) only, "total count" means radioactive solution (made in the 3rd step of the assay protocol) in the assay tube, "added amount" means added radioactive solution (made in the 3rd step of the assay protocol) and "blank" right below "added amount" means empty tubes. These data was taken by 4 tubes each. The most right column shows the average of the 4 values. The sentence at the middle of this page describes how to calculate the Bound [%] values. The "Bound [%]" means binding percentage of radioactive ligand ( $[26,27\text{-methyl}^3\text{H}]1\alpha,25(\text{OH})_2\text{VD}_3$ ) to Vitamin D receptor.

The 7th page shows a graph and a table of the radioactivity count of compounds #346, #344 and  $1\alpha,25(\text{OH})_2\text{VD}_3$  (control) solutions at various concentrations at the last step of the assay protocol. The graph is shown in the same format as the graph on the 4th page, and the table is shown in the same format as the table on the 5th page.

The 8th page shows the method for calculating the binding ratio from the radioactivity data. First I calculated the average values of "blank" and "0" (218 and 2980). Then I described how to calculate the Bound [%] values. In the open bracket, I calculated the radioactive ligand ( $[26,27\text{-methyl}^3\text{H}]1\alpha,25(\text{OH})_2\text{VD}_3$ ) amount in the assay tubes from "total count" values (19 pg / tube). Then I calculated added radioactive ligand from "added amount" values (24 pg / tube). Then I speculated as to why there was a difference between the values for the "total count" and the "added amount."

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The 9th page shows a graph and a table of the binding ratio of compounds #346, #344 and  $1\alpha,25(\text{OH})_2\text{VD}_3$  (control) at various concentrations. The binding ratio was calculated by the method described in the 8th page. In the graph, the concentration value (X-axis) of the compounds #346, #344 and  $1\alpha,25(\text{OH})_2\text{VD}_3$  at 50% bound (Y-axis) are 10-20 pg, 1-2 pg and 20 pg. These data show the binding affinity of compound #344 / #346 is 10-12 / 1-1.8 fold stronger than that of  $1\alpha,25(\text{OH})_2\text{VD}_3$ .

Chart 3 is dated prior to March 17, 1997. Chart 3 is an output of the liquid scintillation counter mentioned on the 3rd page of Note 3. The data were transferred to the 5th and 6th pages of Note 3. The upper half side of the 1st page shows the settings of the scintillation counter. The bottom side of the 1st page and 2nd page shows raw data. The most left column shows tube number, the 3rd column shows the period of measuring (2 min) and the 5th column shows the radioactivity count (unit is dpm).

Exhibit 2 is a set of copies of handouts that I distributed at the group seminar referred to above. The handouts consist of 2 parts. As described at the top of the 1st page of the handout the first part relates to Synthesis of 2-methyl-20epi  $1\alpha,25(\text{OH})_2\text{VD}_3$ .

Page 1 shows the synthesis scheme for compounds #346 and #344, i.e. compounds (68) and (72) respectively. The 1st arrow pointing from vitamin  $\text{D}_2$  to compound 1 indicates that I made compound 1 from vitamin  $\text{D}_2$  by treatment of vitamin  $\text{D}_2$  with  $\text{O}_3$  (Ozone) and  $\text{NaBH}_4$  (Sodium borohydride). The 2nd arrow pointing from compound 1 to compound 2 indicates that I made compound 2 from compound 1 by treatment of compound 1 with  $\text{TsCl}$  (Tosyl chloride) in pyridine (Yield 86%). The 3rd arrow pointing from compound 2 to compound 3 indicates that I made compound 3 from compound 2 by treatment of compound 2 with  $\text{TBSOTf}$  (tert-Butyldimethylsilyl triflate) in 2,6-lutidine (Yield 96%). The 4th arrow pointing from compound 3 to compound 4 indicates that I made compound 4 from compound 3 by treatment of compound 3 with  $\text{DMSO}$  (Dimethylsulphoxide) and  $\text{NaHCO}_3$  (Sodium bicarbonate) (Yield 76%). The 5th arrow pointing from compound 4 to compound in parenthesis indicates that I made the

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compound in parenthesis from compound 4 by treatment of compound 4 with n-Bu<sub>4</sub>NOH (normal-Tetrabutylammonium hydroxide) in CH<sub>2</sub>Cl<sub>2</sub> (Dichloromethane) and H<sub>2</sub>O (water). The 6th arrow pointing from the compound in parenthesis to compound 5 indicates that I made compound 5 from the compound in parenthesis by treatment of the compound in parenthesis with NaBH<sub>4</sub> (Sodium borohydride) and stereoisomer separation by silica gel column chromatography (Yield 45% in 2 steps). The 7th arrow pointing from compound 5 to compound 6 indicates that I made compound 6 from compound 5 by treatment of compound 5 with TsCl (Tosyl chloride) in pyridine (Yield 93%). The 8th arrow pointing from compound 6 to compound 7 indicates that I made compound 7 from compound 6 by treatment of compound 6 with NaI (Sodium iodide) in DMF (Dimethyl formamide) (Yield 92%). The 9th arrow pointing from compound 7 to compound 8 indicates that I made compound 8 from compound 7 and the compound described above the arrow by treatment of both compounds with n-BuLi (normal-Butyl lithium) and HMPA (Hexamethylphosphoramide) in THF (Tetrahydrofuran) (Yield 72% with recovered 28% of starting compound 7. The yield of this step was increased when using distilled HMPA --- 99%). The 10th arrow pointing from compound 8 to compound 9 indicates that I made compound 9 from compound 8 by treatment of compound 8 with Na-Hg (sodium-mercury amalgam) in THF (Tetrahydrofuran) (Yield 64%). The 11th arrow pointing from compound 9 to compound 10 indicates that I made compound 10 from compound 9 by treatment of compound 9 with TsOH (p-Toluenesulfonic acid) (Yield 85%). The 12th arrow pointing from compound 10 to compound 11 indicates that I made compound 11 from compound 10 by treatment of compound 10 with TPAP (Tetrapropylammonium perruthenate), NMO (N-methylmorpholine) and 4ÅMS (molecular sieves 4 angstrom) (Yield 87%). The 13th arrow pointing from compound 11 to compound 12 indicates that I made compound 12 from compound 11 by treatment of compound 11 with Ph<sub>3</sub>P(+)CH<sub>2</sub>Br • Br(-) ((Bromomethyl)triphenylphosphonium bromide) and NaHDMS (Sodium hexamethyldisilazide) (Yield

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The abstract confirms that I had designed and synthesized the 2-methyl-20-epi analogues of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  and had determined the activity of the analogues. In the abstract, compounds (1) and (2) correspond to compounds (72) and (68) in the specification of the above-identified application.

The instructions confirm that the abstract had to be submitted prior to March 17, 1997.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: December 9, 2002

Toshie Fujishima  
Toshie Fujishima

**PATENT APPLICATION**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155

Filed: December 29, 1998



Group Art Unit: 1616

Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

**LIU DECLARATION UNDER 37 C.F.R. § 1.131**

Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Zhaopeng Liu, hereby declare and state:

THAT I am a citizen of P. R. China;

THAT I received a Masters Degree in 1990 from Shandong Medical University, P. R. China and a Ph.D. degree in 2001 from Toyama Medical and Pharmaceutical University, Japan;

THAT I am a member of Japanese Scientific Society related to research in organic chemistry;

THAT I have belonged to Teikyo University, Faculty of Pharmaceutical Sciences, in 1995-1998 as a research fellow, where I have been involved in the synthetic study of Vitamin D<sub>3</sub> in Professor TAKAYAMA's group;

THAT I have been employed by Foreigners Brain Company since 2001, where I have been involved in the synthetic research on new drug candidates.

I have thorough knowledge of the invention in the above-identified patent application, and I have read the non-final Office Action of June 10, 2002 issued in reference to the application. In response to the non-final Office Action, I submit herewith this Declaration, explaining what I observed pertaining to the reduction to practice of the invention claimed in the above-identified application.

Dr. Fujishima was my colleague when I worked in professor TAKAYAMA's group as a research fellow from 1995 to 1998. We understood each other's work very well through daily discussions and regular group seminars. At the time, one of the main research projects of Professor TAKAYAMA's group was the synthetic and biological study of novel 2-substituted Vitamin D<sub>3</sub>. Dr.

Fujishima and I were core members of the project. The compounds in the application are a result of that project.

I recall certain incidents which lead me to remember the author and date of experimental notes (1), (2) and (3) very clearly: Dr. Fujishima planned to present the research results of the 2-substituted Vitamin D<sub>3</sub> project at the Tenth Workshop on Vitamin D, so she had to complete the synthesis and measurement of Vitamin D receptor affinity of the compounds in the application prior to the deadline for submission of presentation abstracts, which was prior to March 17, 1997. Dr. Fujishima presented the results of her work on compounds (68) and (72) in a group seminar held prior to March 17, 1997. Exhibit 2, submitted herewith, comprises copies of the handout that Dr. Fujishima distributed at that seminar. I remember seeing and understanding the handout at the time of the seminar.

Page 1 shows a synthesis scheme for compounds #346 and #344, i.e. compounds (68) and (72) respectively, that had been carried out by Dr. Fujishima or under her supervision. The 1st arrow pointing from vitamin D<sub>2</sub> to compound 1 indicates that compound 1 was made from vitamin D<sub>2</sub> by treatment of vitamin D<sub>2</sub> with O<sub>3</sub> (Ozone) and NaBH<sub>4</sub> (Sodium borohydride). The 2nd arrow pointing from compound 1 to compound 2 indicates that compound 2 was made from compound 1 by treatment of compound 1 with TsCl (Tosyl chloride) in pyridine (Yield 86%). The 3rd arrow pointing from compound 2 to compound 3 indicates that compound 3 was made from compound 2 by treatment of compound 2 with TBSOTf (tert-Butyldimethylsilyl triflate) in 2,6-lutidine (Yield 96%). The 4th arrow pointing from compound 3 to compound 4 indicates that compound 4 was made from compound 3 by treatment of compound 3 with DMSO (Dimethylsulphoxide) and NaHCO<sub>3</sub> (Sodium bicarbonate) (Yield 76%). The 5th arrow pointing from compound 4 to the compound in parenthesis indicates that the compound in parenthesis was made from compound 4 by treatment of compound 4 with n-Bu<sub>4</sub>NOH (normal-Tetrabutylammonium hydroxide) in CH<sub>2</sub>Cl<sub>2</sub> (Dichloromethane) and H<sub>2</sub>O (water). The 6th arrow pointing from the compound in parenthesis to compound 5 indicates that compound 5 was made from the compound in parenthesis by treatment of the compound in parenthesis with NaBH<sub>4</sub> (Sodium borohydride) and stereoisomer separation by silica gel column chromatography (Yield 45% in 2 steps). The 7th arrow pointing from compound 5 to compound 6 indicates that

compound 6 was made from compound 5 by treatment of compound 5 with TsCl (Tosyl chloride) in pyridine (Yield 93%). The 8th arrow pointing from compound 6 to compound 7 indicates that compound 7 was made from compound 6 by treatment of compound 6 with NaI (Sodium iodide) in DMF (Dimethyl formamide) (Yield 92%). The 9th arrow pointing from compound 7 to compound 8 indicates that compound 8 was made from compound 7 and the compound described above the arrow by treatment of both compounds with n-BuLi (normal-Butyl lithium) and HMPA (Hexamethylphosphoramide) in THF (Tetrahydrofuran) (Yield 72% with recovered 28% of starting compound 7. The yield of this step was increased when using distilled HMPA --- 99%). The 10th arrow pointing from compound 8 to compound 9 indicates that compound 9 was made from compound 8 by treatment of compound 8 with Na-Hg (sodium-mercury amalgam) in THF (Tetrahydrofuran) (Yield 64%). The 11th arrow pointing from compound 9 to compound 10 indicates that compound 10 was made from compound 9 by treatment of compound 9 with TsOH (p-Toluenesulfonic acid) (Yield 85%). The 12th arrow pointing from compound 10 to compound 11 indicates that compound 11 was made from compound 10 by treatment of compound 10 with TPAP (Tetrapropylammonium perruthenate), NMO (N-methylmorpholine) and 4ÅMS (molecular sieves 4 angstrom) (Yield 87%). The 13th arrow pointing from compound 11 to compound 12 indicates that compound 12 was made from compound 11 by treatment of compound 11 with  $\text{Ph}_3\text{P}^+(\text{CH}_2\text{Br}) \cdot \text{Br}^-$  ((Bromomethyl)triphenylphosphonium bromide) and NaHDMS (Sodium hexamethyldisilazide) (Yield 57%). The 14th and 15th arrows pointing from compound 12 to 20epi-Ds (compound #344) and 20epi-Aa (compound #346) indicate that 20epi-Ds and 20epi-Aa were made from compound 12 and a TBS protected compound (described in the upper of the page 5 and 6 of the Exhibit 2 (Ref.1)) by treatment of both compounds with  $(\text{dba})_3\text{Pd}_2\text{CHCl}_3$ , (Tris(dibenzylideneacetone)dipalladium(0)-chloroform adduct),  $\text{Ph}_3\text{P}$  (Triphenylphosphine) and  $\text{Et}_3\text{N}$  (Triethylamine) in toluene, and then treatment of the resultant compounds with CSA (Camphor sulfonic acid) in MeOH (methanol).

Therefor, I understood that Dr. Fujishima synthesized compounds (68) and (72) prior to March 17, 1997.

Page 2 shows the method for the VDR binding assay. The resultant binding curves for compounds #346, # 344 and  $1-\alpha, 25-(OH)_2-VD_3$  (control) are also shown. The resultant binding curves of compounds #346, # 344 and  $1\alpha, 25(OH)_2VD_3$  (control) are also shown. The upper half of the page shows the method for VDR binding assay which has the same content as the description in the 1st through 3rd pages of Note 3. The bottom half side of the page shows the resultant binding curves which is the same graph described on the 9th page of Note 3.


Thus, I understood that Dr. Fujishima had confirmed the usefulness of compounds (68) and (72) prior to March 17, 1997.

Pages 3 through 6 show the detailed process for synthesizing compounds #346 and #344 from compound #7 as described in scheme 1 on page 1, accompanied by NMR and MS data of each intermediate compound. As for compounds #346 and #344 i.e. compounds (68) and (72), UV data are also shown. Page 3 shows the detailed process for synthesizing compound 8 from compound 7 and compound 9 from compound 8. Page 4 shows the detailed process for synthesizing compound 10 from compound 9, compound 11 from compound 10 and compound 12 from compound 11. Page 5 shows the detailed process for synthesizing 20epiDs (compound #344) from compound 12 and a TBS protected compound. Page 6 shows the detailed process for synthesizing 20epiAa (compound #346) from compound 12 and a TBS protected compound.

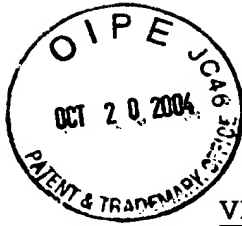
From this I understood that Dr. Fujishima had determined a detailed scheme for how to make compounds (68) and (72) and had identified them prior to March 17, 1997.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 2002.12.10

  
\_\_\_\_\_  
Zhaopeng Liu



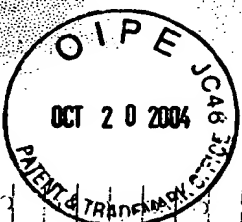


VERIFICATION OF TRANSLATION

I, Kazuya Takenouchi of c/o Teijin Limited, Tokyo Research Center,  
3-2, Asahigaoka 4-chome, Hino-shi, TOKYO, 191-0065 JAPAN,  
hereby certify that to the best of my knowledge and belief the  
attached English translation is a true translation, made by me  
and for which I accept responsibility, of the U.S. patent  
application number 09/214155, filed in the United States on  
November 29, 1998.

This 26<sup>th</sup> day of September, 2002

Kazuya Takenouchi



NO.	PR	INTZ	FREQ(Hz)	POSITION	BAR GRAPH
1	7.51981	0.51025	3006.29	6201	
2	7.49843	0.713021	2997.74	6229	
3	7.40836	0.12475	2961.73	6347	
4	7.40530	0.713811	2930.51	6357	
5	7.40225	0.14321	2939.28	6355	
6	7.39614	0.262821	2936.65	6353	
7	7.39385	0.30828	2935.79	6356	
8	7.27401	0.81013	2902.83	6340	*****
9	7.26103	100.00000	2902.83	6340	*****
10	7.24347	0.52661	2895.81	6263	
11	7.23913	0.77035	2893.68	6370	
12	7.19937	0.76814	2872.51	6340	
13	7.17782	0.42273	2865.97	6649	
14	7.16780	0.44624	2865.60	6652	
15	7.16637	0.50851	2864.99	6664	
16	7.16485	0.47254	2864.38	6565	
17	7.15798	0.35221	2861.63	6675	
18	7.14042	0.17347	2854.61	6588	
19	6.96481	0.56436	2797.24	6684	
20	6.40531	0.93125	2560.79	7251	
21	6.37706	1.07192	2549.49	7268	
22	6.02516	0.98532	2408.75	8155	
23	5.96691	0.83247	2297.46	8194	
24	5.28089	1.51583	2171.21	9134	
25	5.27860	1.65251	2110.29	9137	
26	5.27554	1.75094	2109.07	9161	
27	5.27325	1.62102	2108.15	9144	
28	5.01677	0.75333	2005.62	9460	
29	5.01142	1.99912	2003.48	9467	
30	5.00684	1.89268	2001.65	9493	
31	4.31372	0.71248	1724.55	10201	
32	4.30303	1.15135	1720.20	10415	
33	4.29311	0.75016	1718.31	10478	
34	4.13051	0.16040	1648.31	10641	
35	4.11295	0.12789	1644.39	10641	
36	3.87631	0.20964	1529.68	10654	
37	3.85334	0.23902	1524.97	10674	
38	3.86447	0.46972	1524.47	10921	
39	3.85235	0.38111	1540.75	11000	
40	3.84425	0.63531	1540.22	11005	
41	3.83652	0.39411	1536.87	11016	
42	3.83280	0.51341	1533.81	11026	
43	3.82517	0.34634	1532.29	11031	
44	3.81296	0.23816	1527.43	11041	
45	3.76334	0.10740	1524.35	11057	
46	3.75723	0.10767	1502.08	11130	
47	3.74654	0.24663	1497.80	11144	
48	3.73891	0.15079	1496.89	11147	
49	3.72981	0.18680	1494.75	11154	
50	3.72973	0.13429	1491.09	11166	
51	3.72593	0.16358	1489.56	11171	
52	3.72135	0.14516	1487.73	11177	
53	3.70838	0.15702	1482.54	11194	
54	3.70532	0.20963	1481.32	11198	
55	3.68322	0.21426	1398.93	11448	
56	3.68777	0.19752	1394.35	11483	
57	3.68548	0.22505	1393.43	11486	
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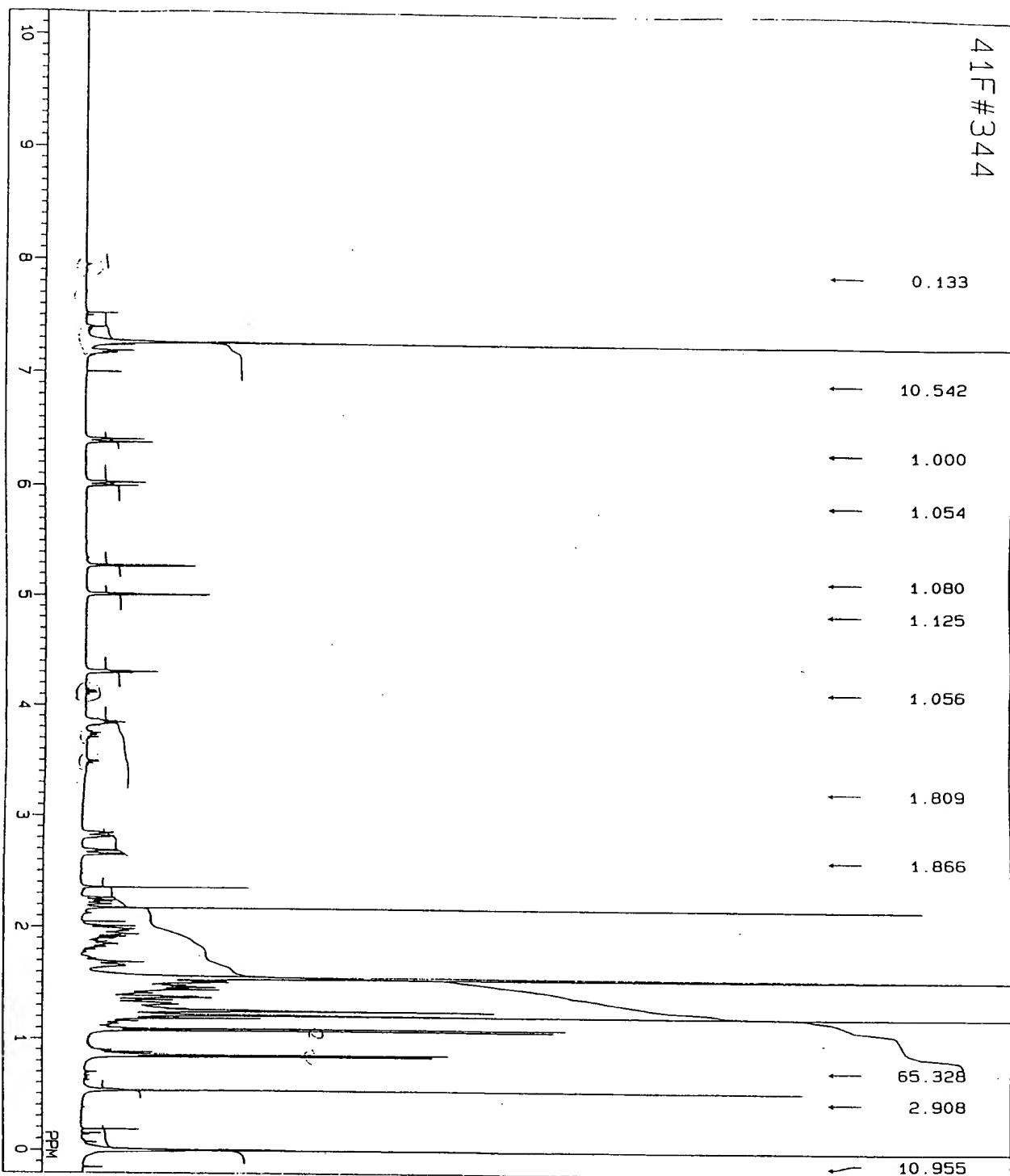
81	1.2476	0.588/3	771.48	13324
82	1.91220	0.53209	764.47	13347
83	1.79949	0.51926	766.80	13359
84	1.87388	0.47665	754.09	13371
85	1.88625	0.42213	754.07	13381
86	1.87633	0.21354	750.12	13394
87	1.86869	0.37384	747.07	13404
88	1.85877	0.31288	743.10	13417
89	1.83947	0.45429	740.97	13424
90	1.84894	0.54529	739.14	13430
91	1.84274	0.55213	734.59	13439
92	1.83663	0.53970	734.25	13447
93	1.82623	0.50725	730.90	13457
94	1.81984	0.36071	727.54	13468
95	1.79770	0.18735	715.59	13477
96	1.71907	0.19456	687.28	13500
97	1.68854	0.97939	675.05	13540
98	1.65229	0.31122	666.75	13560
99	1.65229	0.25825	666.40	13568
100	1.45190	0.57322	620.73	14018
101	1.35268	2.7474697	619.51	14022
102	1.57461	23.68475	608.83	14037
103	1.52489	2.23817	607.30	14062
104	1.51908	2.14206	603.33	14075
105	1.50975	2.30349	601.81	14080
106	1.50534	2.30349	598.75	14091
107	1.49594	1.38147	594.79	14103
108	1.48778	1.63270	590.82	14116
109	1.47786	1.78728	585.63	14135
110	1.46488	2.09681	581.97	14145
111	1.45572	1.76173	579.22	14154
112	1.44885	1.68220	577.09	14161
113	1.44350	2.15006	574.95	14169
114	1.43816	1.24164	572.20	14177
115	1.43129	1.032275	568.24	14180
116	1.42137	0.99560	562.13	14201
117	1.41297	1.10303	558.78	14221
118	1.40610	0.79794	553.69	14237
119	1.39770	0.64852	552.37	14242
120	1.38549	0.82600	549.01	14253
121	1.38167	1.78310	539.86	14283
122	1.37327	2.04219	536.60	14293
123	1.36037	1.81708	530.70	14313
124	1.34974	0.85535	527.65	14333
125	1.32749	1.37920	519.10	14351
126	1.31984	1.38769	516.97	14358
127	1.31164	1.51146	509.34	14378
128	1.29847	1.01536	504.76	14398
129	1.28512	1.05290	501.40	14409
130	1.27312	1.50170	497.74	14421
131	1.27104	1.64288	496.52	14425
132	1.26257	2.70049	491.94	14440
133	1.25419	6.42871	489.23	14462
134	1.24503	2.32149	487.85	14466
135	1.24198	1.52839	484.87	14490
136	1.23053	1.11573	482.06	14532
137	1.22747	1.52209	481.25	14532
138	1.21373	32.59395	478.85	14532
139	1.18778	2.79381	474.85	14532
140	1.16956	0.62643	465.87	14532
141	1.16030	0.87387	463.40	14532
142	1.14324	0.51276	455.85	14532
143	1.13993	0.53358	453.32	14532
144	1.13434	0.55667	451.49	14532
145	1.12442	0.40339	442.81	14532
146	1.10763	0.40489	438.40	14532
147	1.09160	7.52585	422.06	14532
148	1.07480	2.34448	403.44	14730
149	1.05572	0.30532	375.37	14822
150	1.00915	0.11868	372.62	14831
151	0.93893	0.12279	368.04	14846
152	0.92061	0.16872	365.60	14854
153	0.91430	0.13517	358.28	14878
154	0.89628	0.45087	356.75	14883
155	0.87236	0.34908	351.87	14899
156	0.88015	1.10219	344.54	14923
157	0.86183	0.92495	342.10	14931
158	0.85572	5.69947	335.39	14953
159	0.83854	0.41266	325.54	15136
160	0.83854	0.21795	265.81	15181
161	0.69923	0.10733	249.63	15234
162	0.62442	0.10717	213.01	15354
163	0.52582	11.15377	73.24	15812
164	0.18321	0.88836	58.29	15841
165	0.14350	0.29633	27.47	15962
166	0.06870	0.21470	3.05	16042
167	0.00363	83.24671	0.00	16052
168	0.00000	83.24671	-3.36	16053
169	0.00840	83.24671	-3.36	16053
170	0.00893	0.15432	-12.70	16103
171	0.00893	0.15432	-12.70	16103
172	0.00893	0.15432	-12.70	16103

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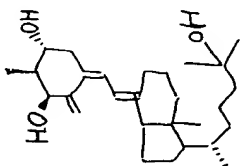
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Exhibit I

10:58:02



SLVNT CDCL3  
 OBNUC 1H  
 OBFRQ 399.65 MHz  
 OBSET 124.00 KHz  
 OBFIN 10905.1 Hz  
 PW1 5.9 us  
 POINT 32768  
 SAMPD 32768  
 SCANS 9216  
 DUMMY 0  
 FREQU 5000.0 Hz  
 FILTR 5000 Hz  
 ACQTM 3.277 sec  
 PD 5.000 sec  
 RGAIN 24  
 BF 0.10 Hz  
 T1 0.0 %  
 T2 0.0 %  
 T3 90.0 %  
 T4 100.0 %  
 EXMOD SGNON  
 DFILE (100,140) FNO344  
 SHMFL TH5  
 SPEED 15 Hz  
 OPERATOR J. SHIMODE



Experimental note of compound synthesis with English translation

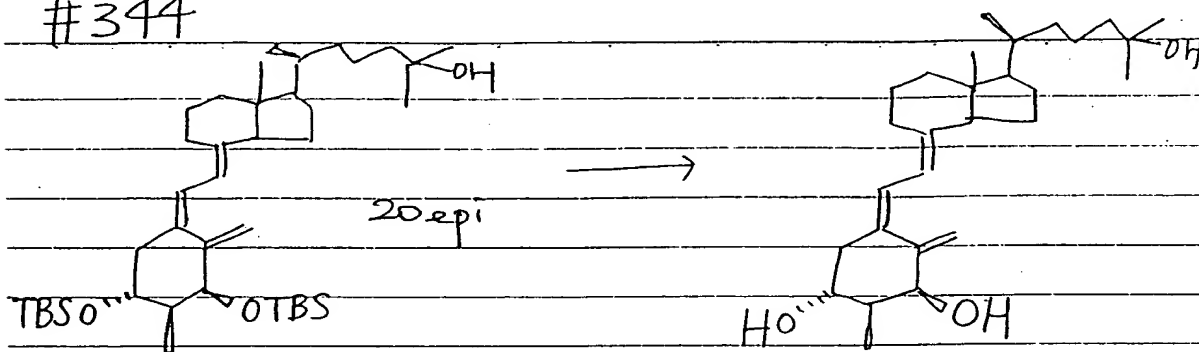
Compound (72) / 20epi Ds / # 344

33.8904  
9005

33.5847  
594

10 mg

#344



MW

MW 430.67

[ protective material ]

{ #343 [本] 体 } work up

CSA MW 232.30 11 mg

MeOH 1 ml

[ stirring under Ar at rt ]

[ Ar下 14:20 ~ ]

9:00

~50 ml

MeOHを留去し、水を加え、EA抽出、brine洗い。

MgSO<sub>4</sub>上脱水、ろ過、エバポレート。

シリカゲルカラム (φ0.9 cm 10 cm height, EA/n-hex = 1:1)

にて精製

9.3 mg (y. 63%)

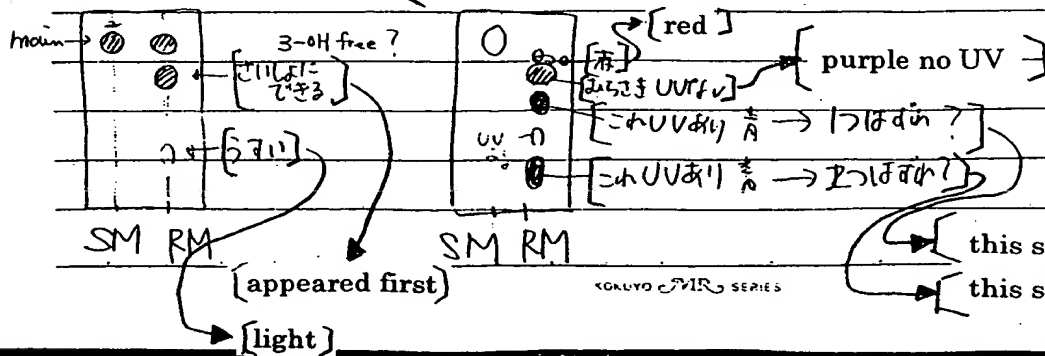
→ HPLCにて分離

MeOH was distilled away, water was added, extracted with EA, washed with dried over MgSO<sub>4</sub>, dehydration, filtration, evaporation purification by silica-gel column chromatography

(φ 0.9 cm 10 cm height, EA/n-Hex = 1:1)

→ Separation by HP.

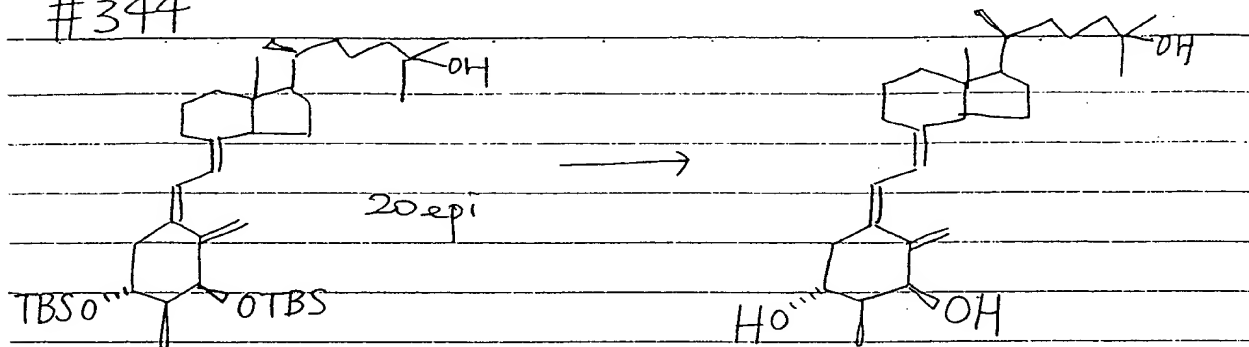
EA/n-hex = 1:1



33.8904  
92.25  
30.2

33.5847  
594  
10mg

#344



MW

MW 430.67

#343 ホルモン work up

CSA MW 232.30 11mg  
MeOH 1ml

Art 14-20 ~  
~50ml

9:00

MeOHを留去し、水を加え、EA抽出、brine洗う。

MgSO<sub>4</sub>上脱水、3カ: エバノレート。

シリカゲルカラム (Φ0.9cm 10cm height, EA=n-hex=1=1)

にて精製

9.3mg (y. 63%)

→ HPLCにて分離

EA=n-hex=1=1

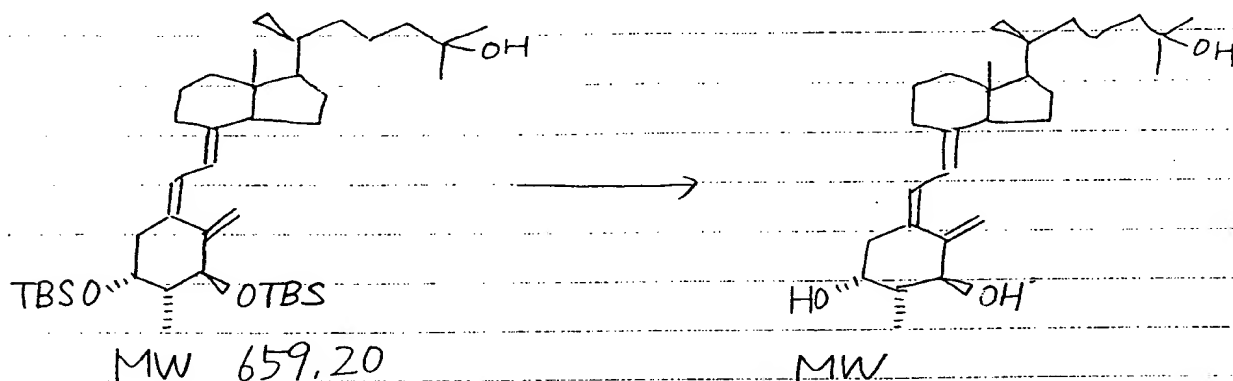
<p>h<sub>max</sub> →</p>	<p>3-OH free?</p> <p>こいれにできる。</p>		<p>UV -</p> <p>こいれに UV あり</p> <p>こいれに UV あり → 1つはどん?</p> <p>こいれに UV あり → 2つはどん?</p>
<p>SM RM</p>		<p>SM RM</p>	

KUJURO - PIR - SERIES

Exhibit 1  
Note 2

4.5

#346



#345のwork up  
 { CSA  
 MeOH

11mg  
 1ml

20:30~

rtかrt後は 反応液から MeOHを  
 とはし、水を加え EA抽出。  
 brine 洗ひ MgSO<sub>4</sub>上 脱水  
 ろか、エバポ。

11:00

シリカゲルカラムで分離後  
 HPLCカラム (ODS (18)) で分離  
 RP-18

4.5mg  
 (y. 31%)

After stirring at rt, MeOH was evaporated from reaction mixture,  
 water was added and extracted with EA  
 washed with brine, dried over MgSO<sub>4</sub>  
 filtered, evaporated

After separation by silica gel column chromatography  
 separation by HPLC column (ODS (18))

41F#346

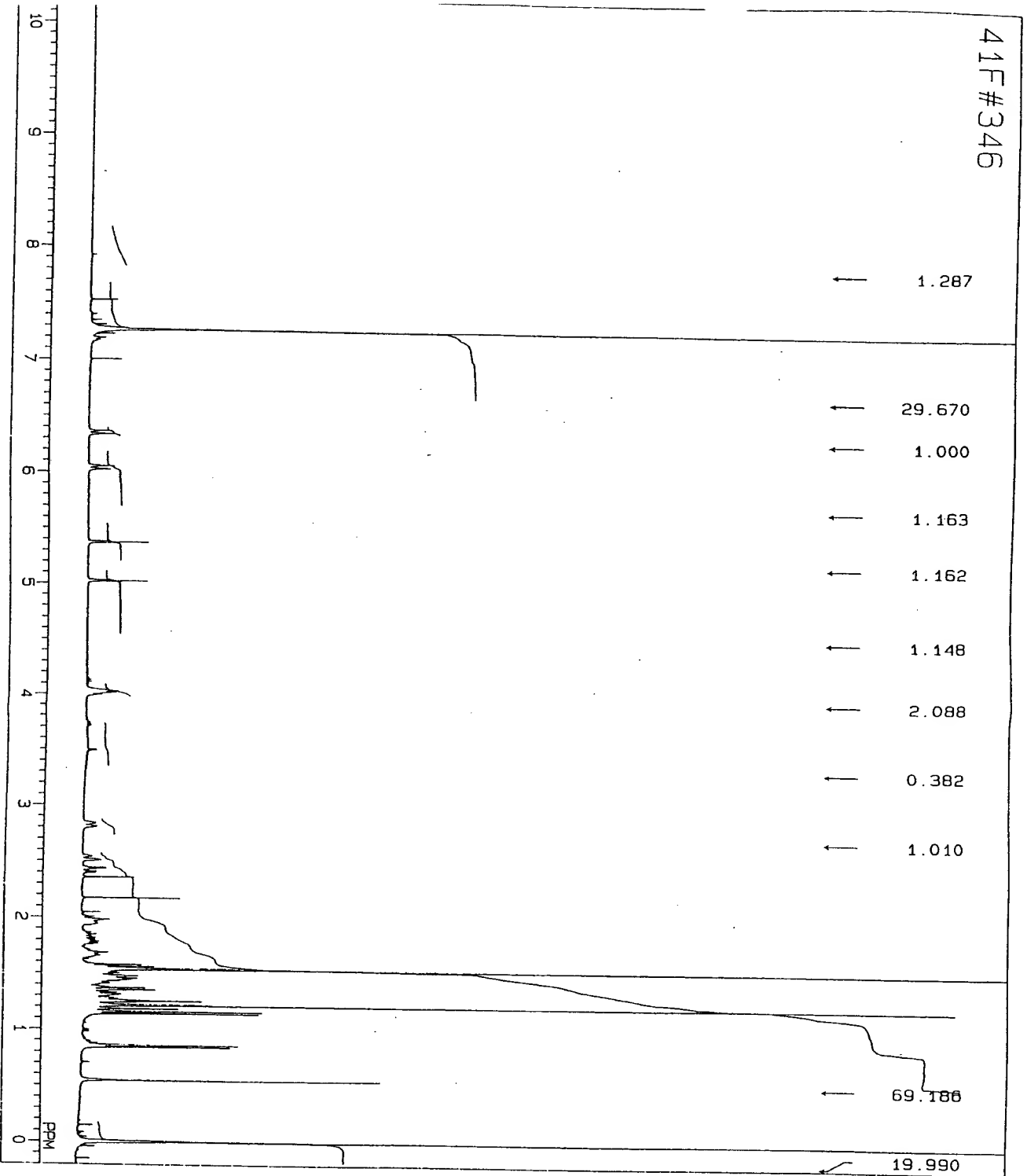


Exhibit 1  
Chart 1, p. 1  
11:26:05

SLVNT CDCL3  
OBNUC 1H  
OBFRQ 399.65 MHz  
OBSET 124.00 kHz  
OBFIN 10905.1 Hz  
PW1 5.9 us  
POINT 32768  
SAMPD 32768  
SCANS 9216  
DUMMY 0  
FREQU 5000.0 Hz  
FILTR 5000 Hz  
ACQTM 3.277 sec  
PD 5.000 sec  
RGAIN 25  
BF 0.10 Hz  
T1 0.0 %  
T2 0.0 %  
T3 90.0 %  
T4 100.0 %  
EXMOD SGNON  
DFILE (100, 140) FNO346  
SHMFL THS  
SPEED 15 Hz  
OPERATOR J.SHIMODE

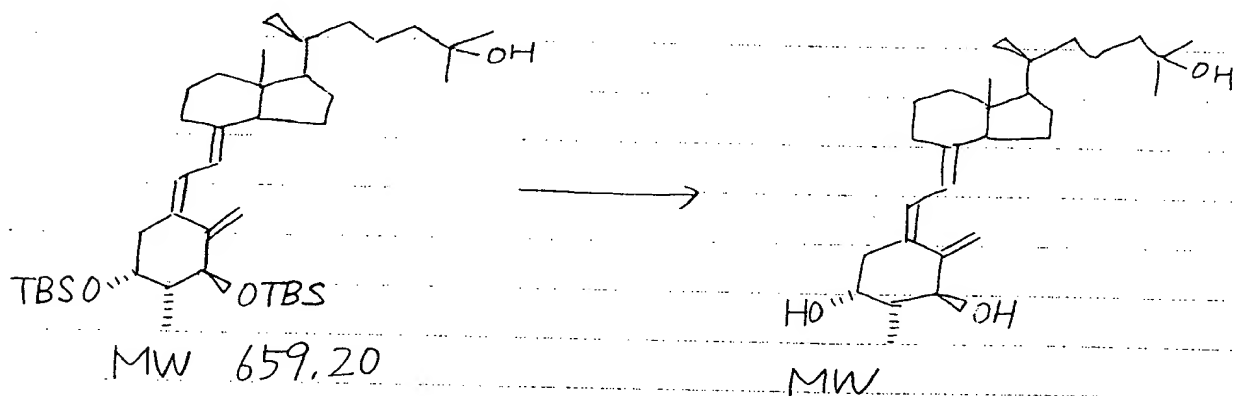
PRM	INTEN	FREQ (MHz)	POSITION	BAR GRAPH
7.91370	0.0733	3163.76	2660	
7.91961	0.0875	3169.76	2660	
7.92057	0.0875	3169.76	2660	
7.92352	0.1211	3169.76	2660	
7.94194	0.20745	3169.76	2660	
7.96199	0.27665	3169.76	2660	
7.96207	1.00.00000	2902.53	6540	
7.92813	0.33301	2893.68	6540	
7.92057	0.43407	2896.66	6592	
7.91849	0.28503	2892.31	6639	
7.91839	0.19619	2894.87	6647	
7.91837	0.18829	2894.99	6663	
7.91798	0.13951	2894.63	6674	
7.91889	0.06443	2894.00	6699	
6.99614	0.56372	2796.94	6686	
6.99396	0.41831	2892.15	7179	
6.93126	0.38337	2817.00	8129	
6.91905	0.35337	2746.44	9011	
6.91402	0.25432	2746.44	9011	
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48	2.1231	0.3213	94.2	22	1.2398	
49	2.1514	0.3407	93.7	34	1.2914	
50	2.1821	0.3602	93.2	46	1.3465	
51	2.2152	0.3808	94.1	77	1.3965	
52	2.2521	0.4026	94.6	53	1.4505	
53	2.2929	0.4255	94.8	53	1.5085	
54	2.3377	0.4495	94.7	87	1.5717	
55	2.3865	0.4746	94.8	60	1.6399	
56	2.4393	0.5008	94.2	92	1.7130	
57	2.4961	0.5281	94.2	24	1.7905	
58	2.5569	0.5565	94.2	47	1.8697	
59	2.6217	0.5860	94.2	79	1.9516	
60	2.6905	0.6166	94.2	53	2.0363	
61	2.7633	0.6483	94.2	53	2.1238	
62	2.8411	0.6811	94.2	53	2.2141	
63	2.9239	0.7150	94.2	53	2.3073	
64	3.0117	0.7500	94.2	53	2.4034	
65	3.1045	0.7861	94.2	53	2.5025	
66	3.2023	0.8233	94.2	53	2.6046	
67	3.3051	0.8616	94.2	53	2.7097	
68	3.4129	0.9010	94.2	53	2.8178	
69	3.5257	0.9415	94.2	53	2.9289	
70	3.6435	0.9832	94.2	53	3.0430	
71	3.7663	1.0261	94.2	53	3.1601	
72	3.8941	1.0702	94.2	53	3.2802	
73	4.0269	1.1155	94.2	53	3.4033	
74	4.1647	1.1620	94.2	53	3.5294	
75	4.3075	1.2097	94.2	53	3.6585	
76	4.4553	1.2586	94.2	53	3.7906	
77	4.6081	1.3087	94.2	53	3.9257	
78	4.7659	1.3600	94.2	53	4.0638	
79	4.9287	1.4125	94.2	53	4.2049	
80	5.0965	1.4662	94.2	53	4.3490	
81	5.2693	1.5211	94.2	53	4.4961	
82	5.4471	1.5772	94.2	53	4.6462	
83	5.6309	1.6345	94.2	53	4.7993	
84	5.8207	1.6930	94.2	53	4.9554	
85	6.0165	1.7526	94.2	53	5.1145	
86	6.2183	1.8133	94.2	53	5.2766	
87	6.4261	1.8751	94.2	53	5.4417	
88	6.6409	1.9380	94.2	53	5.6098	
89	6.8627	2.0020	94.2	53	5.7809	
90	7.0915	2.0671	94.2	53	5.9550	
91	7.3273	2.1333	94.2	53	6.1321	
92	7.5701	2.2006	94.2	53	6.3122	
93	7.8209	2.2690	94.2	53	6.4953	
94	8.0797	2.3385	94.2	53	6.6814	
95	8.3465	2.4091	94.2	53	6.8705	
96	8.6213	2.4808	94.2	53	7.0626	
97	8.9041	2.5535	94.2	53	7.2577	
98	9.1949	2.6273	94.2	53	7.4558	
99	9.4937	2.7021	94.2	53	7.6569	
100	9.8005	2.7780	94.2	53	7.8610	
101	10.1153	2.8549	94.2	53	8.0681	
102	10.4381	2.9328	94.2	53	8.2782	
103	10.7689	3.0117	94.2	53	8.4913	
104	11.1077	3.0916	94.2	53	8.7074	
105	11.4545	3.1725	94.2	53	8.9265	
106	11.8093	3.2544	94.2	53	9.1486	
107	12.1721	3.3373	94.2	53	9.3737	
108	12.5429	3.4212	94.2	53	9.6018	
109	12.9217	3.5061	94.2	53	9.8319	
110	13.3045	3.5919	94.2	53	10.0640	
111	13.6913	3.6787	94.2	53	10.2981	
112	14.0821	3.7665	94.2	53	10.5342	
113	14.4769	3.8553	94.2	53	10.7723	
114	14.8757	3.9451	94.2	53	11.0124	
115	15.2785	4.0359	94.2	53	11.2545	
116	15.6853	4.1277	94.2	53	11.4986	
117	16.0961	4.2205	94.2	53	11.7447	
118	16.5109	4.3143	94.2	53	11.9928	
119	16.9307	4.4091	94.2	53	12.2429	
120	17.3555	4.5049	94.2	53	12.4950	
121	17.7853	4.6017	94.2	53	12.7491	
122	18.2201	4.6995	94.2	53	13.0052	
123	18.6609	4.7983	94.2	53	13.2633	
124	19.1077	4.8981	94.2	53	13.5234	
125	19.5605	4.9989	94.2	53	13.7855	
126	20.0193	5.0997	94.2	53	14.0496	
127	20.4841	5.1995	94.2	53	14.3157	
128	20.9549	5.2993	94.2	53	14.5838	
129	21.4317	5.3991	94.2	53	14.8539	
130	21.9145	5.4989	94.2	53	15.1250	
131	22.4033	5.5987	94.2	53	15.3981	
132	22.8981	5.6985	94.2	53	15.6732	
133	23.3989	5.7983	94.2	53	15.9503	
134	23.9057	5.8981	94.2	53	16.2294	
135	24.4185	5.9979	94.2	53	16.5105	
136	24.9373	6.0977	94.2	53	16.7936	
137	25.4621	6.1975	94.2	53	17.0787	
138	25.9929	6.2973	94.2	53	17.3658	
139	26.5297	6.3971	94.2	53	17.6549	
140	27.0725	6.4969	94.2	53	17.9460	
141	27.6213	6.5967	94.2	53	18.2391	
142	28.1761	6.6965	94.2	53	18.5342	
143	28.7369	6.7963	94.2	53	18.8313	
144	29.3037	6.8961	94.2	53	19.1304	
145	29.8765	6.9959	94.2	53	19.4315	
146	30.4553	7.0957	94.2	53	19.7346	
147	31.0401	7.1955	94.2	53	20.0397	
148	31.6309	7.2953	94.2	53	20.3468	
149	32.2277	7.3951	94.2	53	20.6559	
150	32.8305	7.4949	94.2	53	20.9670	
151	33.4393	7.5947	94.2	53	21.2801	
152	34.0541	7.6945	94.2	53	21.5952	
153	34.6749	7.7943	94.2	53	21.9123	
154	35.3017	7.8941	94.2	53	22.2314	
155	35.9345	7.9939	94.2	53	22.5525	
156	36.5733	8.0937	94.2	53	22.8756	
157	37.2181	8.1935	94.2	53	23.2007	
158	37.8689	8.2933	94.2	53	23.5278	
159	38.5257	8.3931	94.2	53	23.8569	
160	39.1885	8.4929	94.2	53	24.1880	
161	39.8573	8.5927	94.2	53	24.5211	
162	40.5321	8.6925	94.2	53	24.8562	
163	41.2129	8.7923	94.2	53	25.1933	
164	41.8997	8.8921	94.2	53	25.5324	
165	42.5925	8.9919	94.2	53	25.8735	
166	43.2913	9.0917	94.2	53	26.2166	
167	43.9961	9.1915	94.2	53	26.5617	
168	44.7069	9.2913	94.2	53	26.9088	
169	45.4237	9.3911	94.2	53	27.2579	
170	46.1465	9.4909	94.2	53	27.6090	
171	46.8753	9.5907	94.2	53	27.9621	
172	47.6101	9.6905	94.2	53	28.3172	
173	48.3509	9.7903	94.2	53	28.6743	
174	49.0977	9.8901	94.2	53	29.0334	
175	49.8505	9.9899	94.2	53	29.3945	
176	50.6093	10.0897	94.2	53	29.7576	
177	51.3741	10.1895	94.2	53	30.1227	
178	52.1449	10.2893	94.2	53	30.4898	
179	52.9217	10.3891	94.2	53	30.8589	
180	53.7045	10.4889	94.2	53	31.2300	
181	54.4933	10.5887	94.2	53	31.6031	
182	55.2881	10.6885	94.2	53	31.9782	
183	56.0889	10.7883	94.2	53	32.3553	
184	56.8957	10.8881	94.2	53	32.7344	
185	57.7085	10.9879	94.2	53	33.1155	
186	58.5273	11.0877	94.2	53	33.4986	
187	59.3521	11.1875	94.2	53	33.8837	
188	60.1829	11.2873	94.2	53	34.2708	
189	61.0197	11.3871	94.2	53	34.6599	
190	61.8625	11.4869	94.2	53	35.0510	
191	62.7113	11.5867	94.2	53	35.4441	
192	63.5661	11.6865	94.2	53	35.8392	
193	64.4269	11.7863	94.2	53	36.2363	
194	65.2937	11.8861	94.2	53	36.6354	
195	66.1665	11.9859	94.2	53	37.0365	
196	67.0453	12.0857	94.2	53	37.4396	
197	67.9301	12.1855	94.2	53	37.8447	
198	68.8209	12.2853	94.2	53	38.2518	
199	69.7177	12.3851	94.2	53	38.6609	
200	70.6205	12.4849	94.2	53	39.0720	
201	71.5293	12.5847	94.2	53	39.4851	
202	72.4441	12.6845	94.2	53	39.9002	
203	73.3649	12.7843	94.2	53	40.3173	
204	74.2917	12.8841	94.2	53	40.7364	
205	75.2245	12.9839	94.2	53	41.1575	
206	76.1633	13.0837	94.2	53	41.5806	
207	77.1081	13.1835	94.2	53	42.0057	
208	78.0589	13.2833	94.2	53	42.4328	
209	79.0157	13.3831	94.2	53	42.8619	
210	79.9785	13.4829	94.2	53	43.2930	
211	80.9473	13.5827	94.2	53	43.7261	
212	81.9221	13.6825	94.2	53	44.1612	
213	82.9029	13.7823	94.2	53	44.5983	
214	83.8897	13.8821	94.2	53	45.0374	
215	84.8825	13.9819	94.2	53	45.4785	
216	85.8813	14.0817	94.2	53	45.9216	
217	86.8861	14.1815	94.2	53	46.3667	
218	87.8969	14.2813	94.2	53	46.8138	
219	88.9137	14.3811	94.2	53	47.2629	
220	89.9365	14.4809	94.2	53	47.7140	
221	90.9653	14.5807	94.2	53	48.1671	
222	91.9991	14.6805	94.2	53	48.6222	
223	93.0379	14.7803	94.2	53	49.0793	
224	94.0817	14.8801	94.2	53	49.5384	
225	95.1305	14.9799	94.2	53	49.9995	
226	96.1843	15.0797	94.2	53	50.4626	
227	97.2431	15.1795	94.2	53	50.9277	
228	98.3069	15.2793	94.2	53	51.3948	
229	99.3757	15.3791	94.2	53	51.8639	
230	100.4495	15.4789	94.2	53	52.3350	
231	101.5283	15.5787	94.2	53	52.8081	
232	102.6121	15.6785	94.2	53	53.2832	
233	103.7009	15.7783	94.2	53	53.7603	
234	104.7947					



4.5

#346



{ #345のworkup  
 CSA  
 MeOH

11mg  
 1ml

20:30~

1) 反応液から MeOH を  
 とし、水を加え EA 抽出。  
 brine 洗う。MgSO<sub>4</sub> 上 脱水  
 2) エバポ。

11:00

シリカゲルカラムで分離後 ~~4.5mg~~ 4.5mg  
 HPLC カラム (ODS (H)) で分離, (y, 31%)  
 RP-18



Experimental note of VDR binding affinity with English translation  
Compound (68) / 20epi Aa / # 346 and Compound (72) / 20epi Ds / # 344

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Experiment of Bovine Thymus VDR binding affinity (# 7)

- ① Make phosphate-potassium buffer Keeping at 4℃
- ② Diluted solution series of  $1\alpha,25(\text{OH})_2\text{D}_3$ , #344, #346
- ③ Concentration preparation of  $[26,27\text{-methyl}^3\text{H}] 1\alpha,25(\text{OH})_2\text{D}_3$  solution  
Take 100  $\mu\text{L}$  and evaporate Add 6.25 mL of Japanese pharmacopeia grade ethanol
- ④ Pour sample / 50  $\mu\text{L}$  Japanese pharmacopeia grade ethanol (②) into disposable culture tube (12 x 75 mm IWAKI) in concentration order (from thin to dense)  
(like ⑭ ⑳ → ① ⑮)  
⑳ → ㉑ are Japanese pharmacopeia grade ethanol only (by dispenser)
- ⑤ Make receptor solution (lot 110431 YAMASA)  
Pour 5 mL of phosphate-potassium buffer (①) into a vessel containing thymus receptor and dissolve the receptor gently. Add further 50 mL of the buffer and stir gently
- ⑥ Add 500  $\mu\text{L}$  of the receptor solution to each tubes except blank (㉘ ㉙ ㉚ ㉛)  
Add 500  $\mu\text{L}$  of the buffer solution to each blank tube
- ⑦ Stir by vortex, avoid forming
- ⑧ Pre incubate at rt for 1 hr  
Put the top on the tubes by plastic wrap & aluminum foil  
13:40 ~ 14:40 rt approximately 22℃

RI room

⑨ Add 50  $\mu$ L of the hot solution (③) to each tubes by dispenser

In case of hot only count (⑨⑦ ⑨⑧ ⑨⑨ ⑩⑩), hot solution is added to vial tube

⑩ Stir by vortex, avoid forming

⑪ Put the top on the tubes by plastic wrap, put the tubes into 4°C refrigerator in RI room, and stand overnight

15:10~

97	16217.7 dpm	
98	16349.9	
99	16280.0	
100	16634.8	
101	54.3	
102	28.3	
103	42.7	
104	56.9	Average 16370 dpm
		" 45 dpm

Add 10 mL of ACS-II and measure radioactivity count for 1 min by Aloka A  
Stand rt and measure radioactivity count for 2 min tomorrow

$$\left( \begin{array}{l} 16370 \text{ dpm} = 273 \text{ dps} = 273 \text{ Bq} \\ 11.4 \text{ GBq} / \text{mg} \text{ therefore } 24 \text{ pg} / \text{tube} \end{array} \right)$$

~9:25

- ⑫ Put out the yesterday's samples from the refrigerator in RI room and add 200  $\mu$ L of DCC solution (lot M602 YAMASA) to each tubes by dispenser except total count tubes (⑨③ ⑨④ ⑨⑤ ⑨⑥)

Add the buffer solution ① to each total count tubes

- ⑬ Vortex tubes

- ⑭ Stand for 30 min at 4°C                      9:50~10:20

10:30~10:40

- ⑮ Centrifuge at 3000 rpm for 10 min at 0°C

- ⑯ Transfer 500  $\mu$ L of supernatant to 20 mL WHEATON vial

Lay ice on tray and put tube on the ice

[ in concentration order (from thin to dense) ① → ⑭      same pipetter tip  
Change pipetter tip ⑮ → ⑳

- ⑰ Add 9.5 mL of ACS-II to each tubes, shake, and measure radioactivity count (2 min)

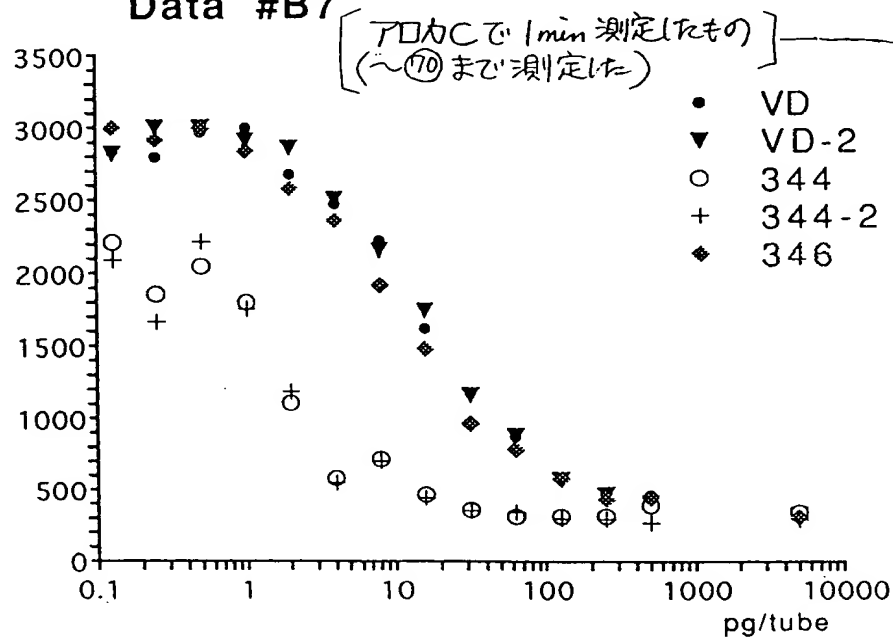
Aloka A

☆ バック  
 ☆ バイアル  
 ☆ カミヤ  
 ☆ センサーマン 1000  
 ☆ " 200

☆ レシーバー  
 ☆ 4.47° に 5.5

No. \_\_\_\_\_  
 Date \_\_\_\_\_  
 ( )

Data #B7



{ This shows the results of 1 min measuring by Aloka C  
 (measured to ~ 70) }

	13	10221				2811			
150µl	10250H <sub>2</sub> VD <sub>3</sub>	#344				#346			
5ng	290 <sup>15</sup>	325 <sup>29</sup>	308 <sup>43</sup>	296 <sup>57</sup>	338 <sup>71</sup>	305 <sup>85</sup>			
500pg	357 <sup>16</sup>	363 <sup>30</sup>	325 <sup>44</sup>	312 <sup>58</sup>	445 <sup>72</sup>	386 <sup>86</sup>			
250	444 <sup>17</sup>	529 <sup>31</sup>	318 <sup>45</sup>	302 <sup>59</sup>	445 <sup>73</sup>	479 <sup>87</sup>			
125	608 <sup>18</sup>	623 <sup>32</sup>	326 <sup>46</sup>	324 <sup>60</sup>	528 <sup>74</sup>	573 <sup>88</sup>			
63	802 <sup>19</sup>	806 <sup>33</sup>	349 <sup>47</sup>	326 <sup>61</sup>	698 <sup>75</sup>	623 <sup>89</sup>			
32	1094 <sup>20</sup>	1166 <sup>34</sup>	391 <sup>48</sup>	387 <sup>62</sup>	1041 <sup>76</sup>	913 <sup>90</sup>			
16	1701 <sup>21</sup>	1676 <sup>35</sup>	458 <sup>49</sup>	369 <sup>63</sup>	1395 <sup>77</sup>	1357 <sup>91</sup>			
8	2164 <sup>22</sup>	2109 <sup>36</sup>	658 <sup>50</sup>	663 <sup>64</sup>	1834 <sup>78</sup>	1822 <sup>92</sup>			
4	2494 <sup>23</sup>	2511 <sup>37</sup>	568 <sup>51</sup>	520 <sup>65</sup>	2428 <sup>79</sup>	2180 <sup>93</sup>			
2	2519 <sup>24</sup>	2536 <sup>38</sup>	1145 <sup>52</sup>	1161 <sup>66</sup>	2766 <sup>80</sup>	2499 <sup>94</sup>			
1	2879 <sup>25</sup>	2768 <sup>39</sup>	1739 <sup>53</sup>	1819 <sup>67</sup>	2768 <sup>81</sup>	2763 <sup>95</sup>			
0.5	2862 <sup>26</sup>	2924 <sup>40</sup>	2081 <sup>54</sup>	2062 <sup>68</sup>	2762 <sup>82</sup>	2768 <sup>96</sup>			
0.25	2851 <sup>27</sup>	2959 <sup>41</sup>	1942 <sup>55</sup>	1847 <sup>69</sup>	2910 <sup>83</sup>	2834 <sup>97</sup>			
0.13	2839 <sup>28</sup>	2690 <sup>42</sup>	1987 <sup>56</sup>	1932 <sup>70</sup>	2990 <sup>84</sup>	2694 <sup>98</sup>			

EXHIBIT 1

Exhibit 1  
Note 3, p. 5  
(Translation)

0	<sup>85</sup> 2744	<sup>86</sup> 2982	<sup>87</sup> 3149	<sup>88</sup> 3048	2980
blank	<sup>89</sup> 224	<sup>90</sup> 166	<sup>91</sup> 174	<sup>92</sup> 311	218
total count	<sup>93</sup> 7965	<sup>94</sup> 8280	<sup>95</sup> 8052	<sup>96</sup> 8325	8155
[入射量]	<sup>97</sup> 16184	<sup>98</sup> 15926	<sup>99</sup> 16360	<sup>100</sup> 16561	16257
blank	<sup>101</sup> 27	<sup>102</sup> 59	<sup>103</sup> 43	<sup>104</sup> 34	40

[ added amount ]

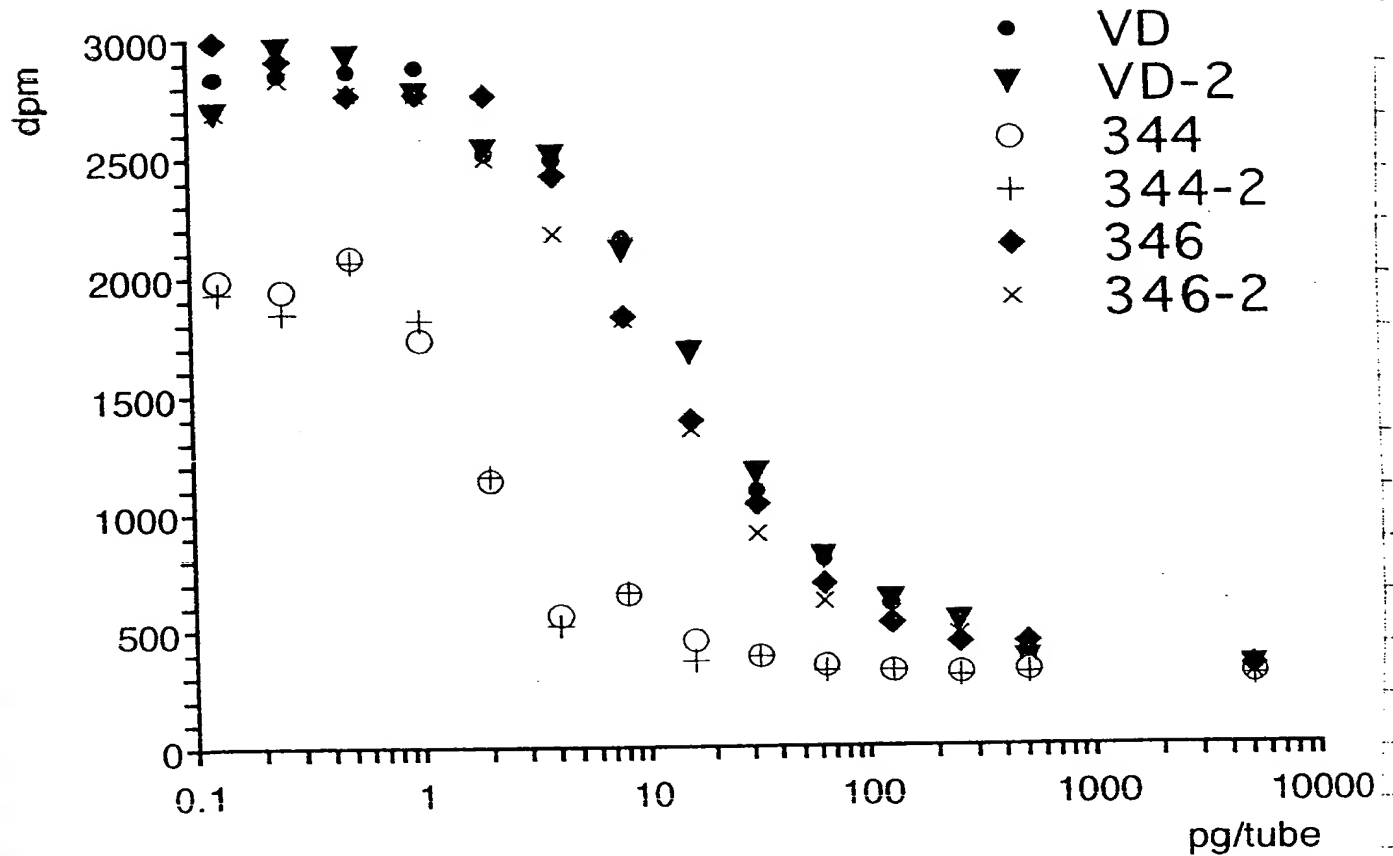
2762

すべての実験値から 218 を引いて  $(2980 - 218)$  で  
割り  $\times 100$  して Bound [%] を計算

Bound [%] was calculated as follows: Subtract 218 from all experimental values, then  
this value divides by  $(2980 - 218)$  and multiply 100.

$$50 + 500 + \frac{50}{200}$$

#B7



	pg/tube	VD	VD-2	344	344-2	346	346-2
0	5000.0	290.00	325.00	308.000	296.00	338.00	305.00
1	500.00	357.00	363.00	325.000	312.00	445.00	386.00
2	250.00	444.00	529.00	318.000	302.00	445.00	477.00
3	125.00	608.00	623.00	326.000	324.00	528.00	573.00
4	63.000	802.00	806.00	349.000	326.00	698.00	623.00
5	32.000	1094.0	1166.0	391.000	387.00	1041.0	913.00
6	16.000	1701.0	1676.0	458.000	369.00	1395.0	1357.0
7	8.0000	2164.0	2109.0	658.000	663.00	1834.0	1822.0
8	4.0000	2494.0	2511.0	568.000	520.00	2428.0	2180.0
9	2.0000	2519.0	2536.0	1145.00	1161.0	2766.0	2499.0
10	1.0000	2879.0	2768.0	1739.00	1819.0	2768.0	2763.0
11	0.50000	2862.0	2924.0	2081.00	2062.0	2762.0	2768.0
12	0.25000	2851.0	2959.0	1942.00	1847.0	2910.0	2834.0
13	0.13000	2839.0	2690.0	1987.00	1932.0	2990.0	2694.0

dpm



<Results>

$$\text{blank} = 224 + 166 + 174 + 311 / 4 = 218$$

$$0 = 2744 + 2982 + 3149 + 3048 / 4 = 2980$$

Bound[%] was calculated as follows: Subtract 218 which is average value of blank from all experimental values, then this value divides by (subtract 218 from 2980 which is average value of drug 0)  $(2980 - 218 = 2762)$  and multiply 100

$$\text{total count} = 7965 + 8280 + 8052 + 8325 / 4 = 8155 \text{ dpm}$$

$8155 / 60 \text{ dps} = 136 \text{ Bq}$  As I put 500  $\mu\text{L}$  from 800  $\mu\text{L}$  and measured radioactivity count

$$136 \times 8 / 5 = 217 \text{ Bq}$$

$$11.4 \text{ GBq} / \text{mg} \text{ therefore } 19 \text{ pg} / \text{tube} \quad \text{J}$$

As average added amount is 16257 dpm

from 271 Bq

$$24 \text{ pg} / \text{tube} \quad \text{J}$$

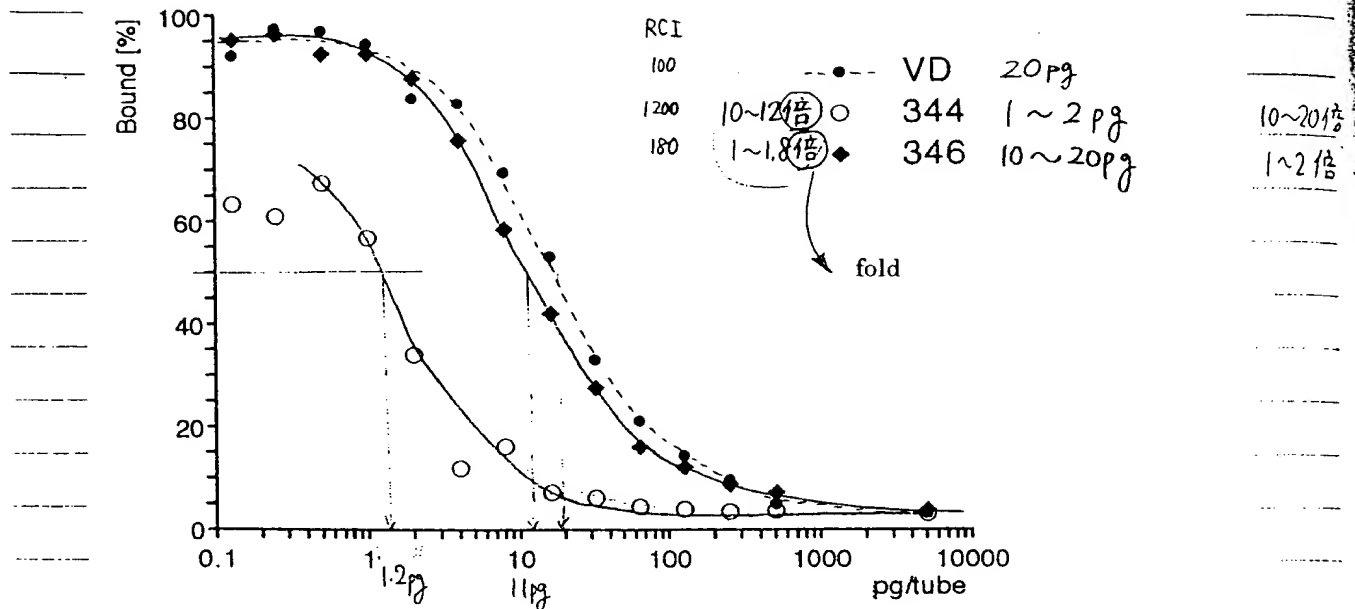
Approximately 80% of hot receptor exists in solution  
and the rest should absorb an inside wall of glass tube

$$\begin{aligned} 217 \text{ Bq} / \text{tube} &= 217 / 4.85 \text{ T} / (50 + 500 + 50) \mu\text{L} \\ &= 0.075 \text{ nM} \end{aligned}$$

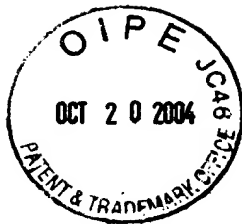
Or, it may exists as  $1\alpha 25(\text{OH})_2$  and the rest may count of decompose stuff

Bovine  
Chicken

#B7(edit)



pg/tube	VD	VD-2	VD-	344	344-2	344-	346	346-2	346-
5000.0	2.6068	3.8740	3.2404	3.25851	2.8240	3.0413	4.3447	3.1499	3.7473
500.00	5.0326	5.2498	5.1412	3.87400	3.4033	3.6387	8.2187	6.0825	7.1506
250.00	8.1825	11.260	9.7212	3.62056	3.0413	3.3309	8.2187	9.3773	8.7980
125.00	14.120	14.663	14.392	3.91021	3.8378	3.8740	11.224	12.853	12.038
63.000	21.144	21.289	21.217	4.74294	3.9102	4.3266	17.379	14.663	16.021
32.000	31.716	34.323	33.020	6.26358	6.1188	6.1912	29.797	25.163	27.480
16.000	53.693	52.788	53.240	8.68936	5.4671	7.0782	42.614	41.238	41.926
8.0000	70.456	68.465	69.461	15.9305	16.112	16.021	58.508	58.074	58.291
4.0000	82.404	83.020	82.712	12.6720	10.934	11.803	80.014	71.035	75.525
2.0000	83.309	83.925	83.617	33.5626	34.142	33.852	92.252	82.585	87.419
1.0000	96.343	92.324	94.334	55.0688	57.965	56.517	92.324	92.143	92.234
0.50000	95.728	97.972	96.850	67.4511	66.763	67.107	92.107	92.324	92.216
0.25000	95.329	99.240	97.285	62.4185	58.979	60.699	97.466	94.714	96.090
0.13000	94.895	89.500	92.198	64.0478	62.056	63.052	100.36	89.645	95.004



## Bovine Thymus VDR への結合実験 (井7)

- ① リン酸カリバリアンを作製 4℃保存
- ②  $1\alpha,25(\text{OH})_2\text{VD}_3$ , #344, #346 の希釈系列
- ③  $[26,27\text{-methyl } 3\text{H}]1\alpha,25(\text{OH})_2\text{VD}_3$  の濃度調整  
100  $\mu\text{l}$  とって とぼし 6.25 ml の局エタ
- ④ disposable culture tube (12x75 mm イワキ) に  
sample / 50  $\mu\text{l}$  局エタ (②) を うすい順に入れて  
( ④⑤ → ①⑤ のように )  
⑤ → ⑥ は 局エタのみ. (分注器で)
- ⑤ レセプタ溶液をつくる (lot 11043) ヤマサ  
Thymus Receptor の容器に リン酸カリバリアン①を  
5 ml 加えて 静かにとくす. さらに 50 ml を  
加えて 静かにまぜる.
- ⑥ レセプタ溶液 500  $\mu\text{l}$  を blank (⑧⑨⑩⑪⑫)  
以外の tube 1 に加える.  
加えたばかりの tube 1 には buffer を 500  $\mu\text{l}$  加える
- ⑦ vortex で あわだて 2 分 15 秒 にかはんする
- ⑧ rt で 1 hr pre incubation  
ラック & ホイルで 3 分 rt 22℃ 5 分  
13:40 ~ 14:40

RI室

- ⑨ hot 溶液 (3) をすべての tube 1 に分注器で  
50  $\mu$ l ずつ加える。  
hot のみ count (97) (98) (99) (100) には  
バイアルに入れる。

- ⑩ vortex で あわだてないようにかくはんする

- ⑪ ラックでふたをして 4°C の RI 室の冷蔵庫に入れ  
over night. 15-10 ~

97	16217.7 dpm
98	16349.9
99	16280.0
100	16634.8
101	54.3
102	28.3
103	42.7
104	56.9

平均 16370 dpm  
45 dpm

10ml の ACS-II を加えて アロカ A で 1 min  
count する。  
10 ml で放置し 次の日に 10 ml には 2 min  
count.

$$\left( \begin{array}{l} 16370 \text{ dpm} = 273 \text{ dps} = 273 \text{ Bq} \\ 11.4 \text{ GBq} / \text{mg} \text{ から } 24 \text{ pg/tube} \end{array} \right)$$

遠心 0°C スイッチ on

~ 9:25 RI室の

⑫ 前日のサンプルを冷蔵庫から出して total count  
(93) (94) (95) (96) 以外の tube に DCC 液を  
(Lot M602 ヤマサ) 200  $\mu$ l ずつ 分注器で加える  
加えなかった tube には ① のバッファを加える

⑬ tube を vortex

⑭ 4°C で 30 min 放置 9:50 ~ 10:20

⑮ 遠心 0°C 10 min 3000 rpm 10:30 ~ 10:40

⑯ 上澄を 500  $\mu$ l ずつ WHEATON の 20ml の  
バイアルに移す バットの底に 2 (CEL) してしける  
（うすい川原に ① → ⑭ チップ 0 同し  
チップ 0 加えて ⑮ → ⑳

⑰ ACS-II を 9.5ml ずつ加えて shake し  
count (2 min) する アロカ A

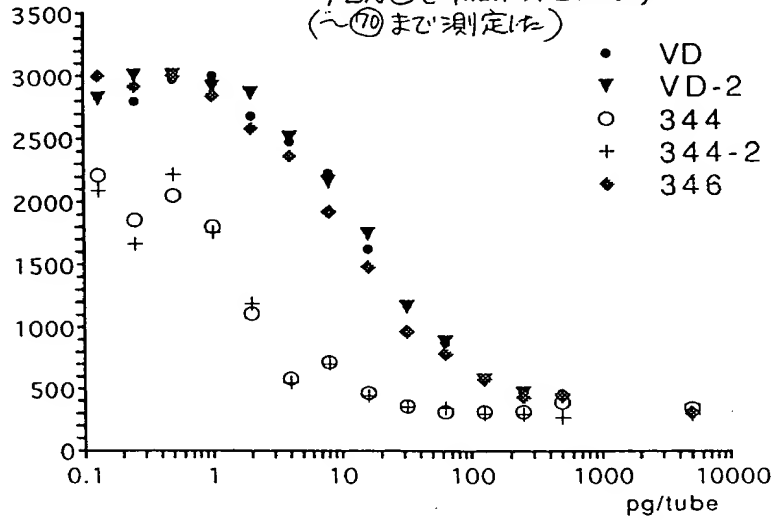
☆ バック  
 ☆ バイアル  
 ☆ カメラ  
 ☆ センサーマン 1000  
 ☆ " 200

☆ レンズ  
 ☆ 4.47° たん

No. \_\_\_\_\_  
 Date \_\_\_\_\_  
 ( )

Data #B7

アロカCで1min測定したもの  
 (〜⑦⑩まで測定した)



	L3			T1221			2121		
150ul	10(250H) <sub>2</sub> VD <sub>3</sub>			#344			#346		
5ng	290	325	29	308	296	57	338	305	
500pg	357	363	30	325	312	58	445	386	
250	444	529	31	318	302	59	445	477	
125	608	623	32	326	324	60	528	573	
63	802	806	33	349	326	61	698	623	
32	1094	1166	34	391	387	62	1041	913	
16	1701	1676	35	458	369	63	1395	1357	
8	2164	2109	36	658	663	64	1834	1822	
4	2494	2511	37	568	520	65	2428	2180	
2	2519	2536	38	1145	1161	66	2766	2499	
1	2879	2768	39	1739	1819	67	2768	2763	
0.5	2862	2924	40	2081	2062	68	2762	2768	
0.25	2851	2959	41	1942	1847	69	2910	2834	
0.13	2839	2690	42	1987	1932	70	2990	2694	

EXHIBIT 1

Exhibit 1  
Note 3, p. 5

0	<sup>85</sup> 2744	<sup>86</sup> 2982	<sup>87</sup> 3149	<sup>88</sup> 3048	2980
blank	<sup>89</sup> 224	<sup>90</sup> 166	<sup>91</sup> 174	<sup>92</sup> 311	218
total count	<sup>93</sup> 7965	<sup>94</sup> 8280	<sup>95</sup> 8052	<sup>96</sup> 8325	8155
入力量	<sup>97</sup> 16184	<sup>98</sup> 15926	<sup>99</sup> 16360	<sup>100</sup> 16561	16257
blank	<sup>101</sup> 27	<sup>102</sup> 59	<sup>103</sup> 43	<sup>104</sup> 34	40

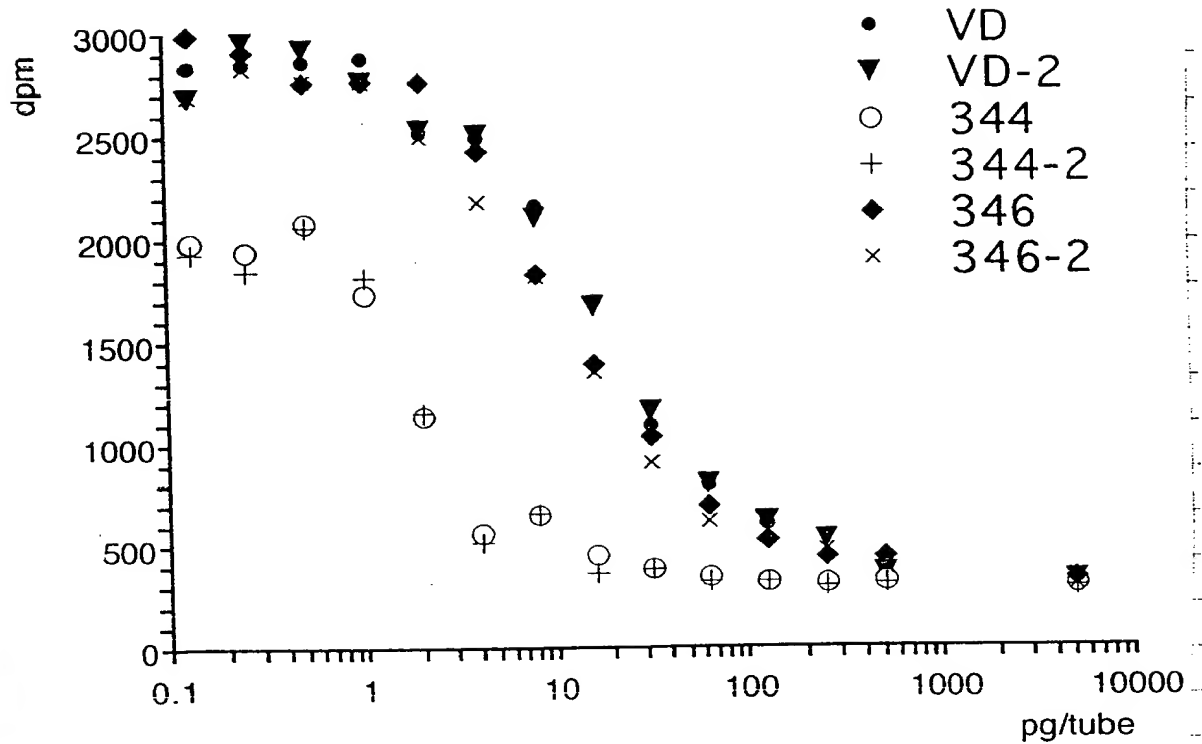
2762

すばの実験値から 218 を引いて (2980-218) を  
割り  $\times 100$  して Band [%] を得た

$$\frac{50}{50 + 500 + 200}$$



#B7



	pg/tube	VD	VD-2	344	344-2	346	346-2
0	5000.0	290.00	325.00	308.000	296.00	338.00	305.00
1	500.00	357.00	363.00	325.000	312.00	445.00	386.00
2	250.00	444.00	529.00	318.000	302.00	445.00	477.00
3	125.00	608.00	623.00	326.000	324.00	528.00	573.00
4	63.000	802.00	806.00	349.000	326.00	698.00	623.00
5	32.000	1094.0	1166.0	391.000	387.00	1041.0	913.00
6	16.000	1701.0	1676.0	458.000	369.00	1395.0	1357.0
7	8.0000	2164.0	2109.0	658.000	663.00	1834.0	1822.0
8	4.0000	2494.0	2511.0	568.000	520.00	2428.0	2180.0
9	2.0000	2519.0	2536.0	1145.00	1161.0	2766.0	2499.0
10	1.0000	2879.0	2768.0	1739.00	1819.0	2768.0	2763.0
11	0.50000	2862.0	2924.0	2081.00	2062.0	2762.0	2768.0
12	0.25000	2851.0	2959.0	1942.00	1847.0	2910.0	2834.0
13	0.13000	2839.0	2690.0	1987.00	1932.0	2990.0	2694.0

Exhibit 1  
Note 3, p. 7

# B2 88% 1/2 B7 80%  
~~#~~ B3 84% 1/19 97/30  
~~#~~ B4 84% 1/2

## ＜結果＞

$$\text{blank} = \frac{224 + 166 + 174 + 311}{4} = 218$$

$$0 = \frac{2744 + 2982 + 3149 + 3048}{4} = 2980.$$

coldon

(すべての実験値から blank の平均値 218 を引いて drug 0 のときの平均 2980 から 218 をひいたもの  $(2980 - 218 = 2762)$  で除し 100 をかけ 総合格率を計算した。

$$\begin{aligned} \text{total count} &= \frac{17965 + 8280 + 8052 + 8325}{4} \\ &= 8155 \text{ dpm} \end{aligned}$$

$$\frac{8155}{60} = 136 \text{ Bq}$$

800  $\mu$ l + 500  $\mu$ l  $\rightarrow$  count 1K of 2"  
 $136 \times \frac{8}{5} = 217 \text{ Bq}$

11.4 GBq/mg 100% 19 pg/tube

入れた量の平均は  $16257 \text{ dpm}$  であるので

271 Bq (5')

24 pg<sup>0</sup>/tube

80%くらいが溶液中に存在し

あとはガラス壁等に吸着していると考えられる。

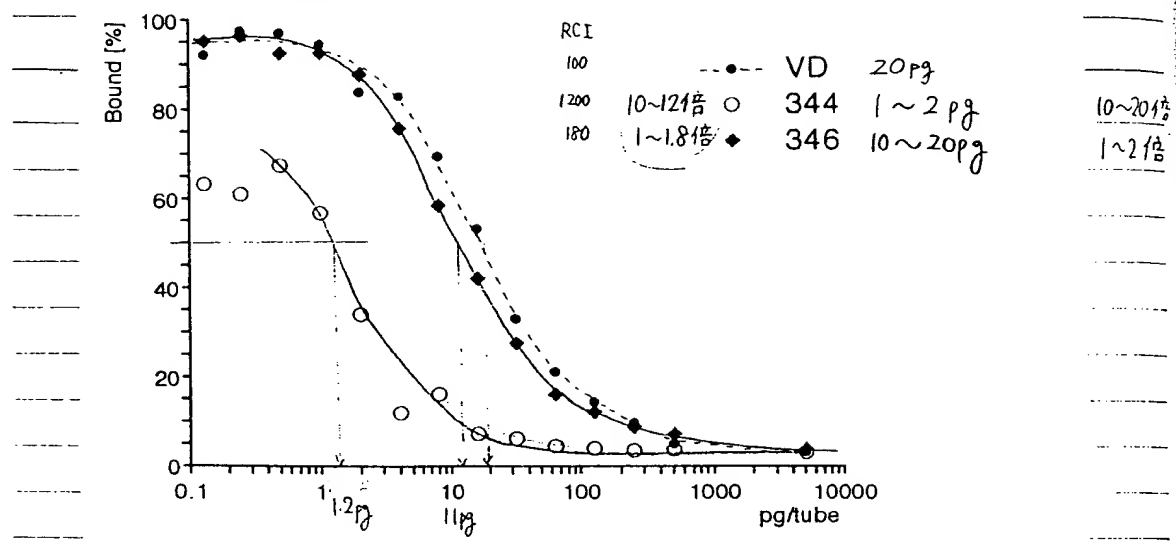
$$217 \text{ Bq/tube} = \frac{217}{4.85 \text{ T} \cdot (50 + 500 + 50)_{\mu\text{L}}} \cdot 10^6$$

$$= 0.075 \text{ nM}$$

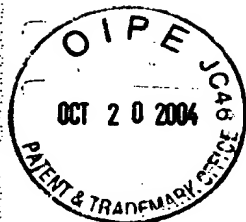
又は 1x25Hz になっているものは、こたれたものの count かもしれない

Bovine  
Chicken

#B7(edit)



pg/tube	VD	VD-2	VD-	344	344-2	344-	346	346-2	346-
5000.0	2.6068	3.8740	3.2404	3.25851	2.8240	3.0413	4.3447	3.1499	3.7473
500.00	5.0326	5.2498	5.1412	3.87400	3.4033	3.6387	8.2187	6.0825	7.1506
250.00	8.1825	11.260	9.7212	3.62056	3.0413	3.3309	8.2187	9.3773	8.7980
125.00	14.120	14.663	14.392	3.91021	3.8378	3.8740	11.224	12.853	12.038
63.000	21.144	21.289	21.217	4.74294	3.9102	4.3266	17.379	14.663	16.021
32.000	31.716	34.323	33.020	6.26358	6.1188	6.1912	29.797	25.163	27.480
16.000	53.693	52.788	53.240	8.68936	5.4671	7.0782	42.614	41.238	41.926
8.0000	70.456	68.465	69.461	15.9305	16.112	16.021	58.508	58.074	58.291
4.0000	82.404	83.020	82.712	12.6720	10.934	11.803	80.014	71.035	75.525
2.0000	83.309	83.925	83.617	33.5626	34.142	33.852	92.252	82.585	87.419
1.0000	96.343	92.324	94.334	55.0688	57.965	56.517	92.324	92.143	92.234
0.50000	95.728	97.972	96.850	67.4511	66.763	67.107	92.107	92.324	92.216
0.25000	95.329	99.240	97.285	62.4185	58.979	60.699	97.466	94.714	96.090
0.13000	94.895	89.500	92.198	64.0478	62.056	63.052	100.36	89.645	95.004



#B7

PODA

2min

BY NO. 2: (H-S DPM ESCR 2min)

15:07

CYCLE : 1

[ 1 ] PRESET TIME (Min.) 2.0  
[ 2 ] REPEAT 1  
[ 3 ] CYCLE 1  
[ 4 ] DATA DPM  
[ 5 ] ISOTOPE H  
[ 6 ] B.K.G. SUB NO  
[ 7 ] HEAD PRINT YES

\* FUNCTION MODE \*

[ 1 ] STANDARDIZATION ESCR  
[ 2 ] CURVE AUTO  
[ 3 ] REJECT NO  
[ 4 ] ESCR PRESET TIME (Min.) 0.4  
[ 5 ] CONSTANT RATIO NO  
[ 6 ] CLEAR CHECK NO  
[ 7 ] 2% ERROR NO  
[ 8 ] FORMATTING NO  
[ 9 ] FILE NO  
[10] REPEAT REPLICATE NO  
[11] AWS YES  
[12] QUENCHING LEVEL AUTO  
[13] BEQUEREL NO  
[14] HALF LIFE NO  
[15] CALCULATION NO  
[16] HISTOGRAM NO

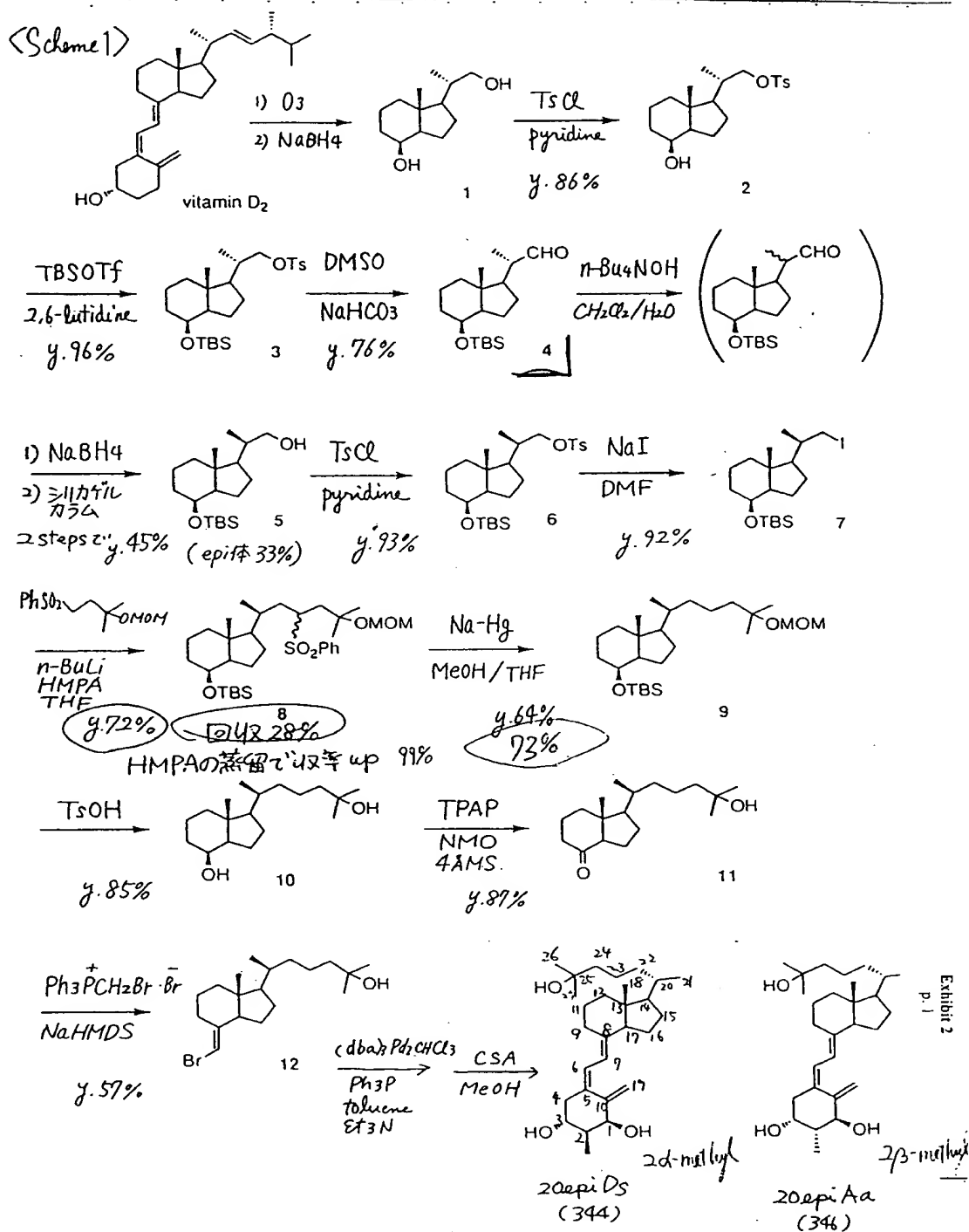
CURVE NO. = 3

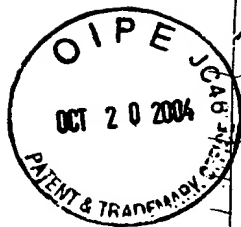
LOW ENERGY G:N A= -0.00789 B= 0.41072 C= 0.45704 D=-124.77292  
LOW ENERGY B:H A= 0.00660 S= 0.20719 C= 0.42623 D= -7.11626

TIME	H-CPM	H-DPM	H-EFF
1 26.24 2.0	80.5	250.6	27.70
2 26.18 2.0	97.5	357.9	27.24
3 26.20 2.0	121.5	444.1	27.36
4 26.24 2.0	168.0	608.9	27.59
5 26.22 2.0	220.5	802.6	27.47
6 26.20 2.0	299.5	1094.7	27.36
7 26.22 2.0	467.5	1701.8	27.47
8 26.26 2.0	599.5	2164.0	27.70
9 26.20 2.0	682.5	2494.5	27.36
10 26.24 2.0	695.0	2519.1	27.59
11 26.24 2.0	794.5	2879.8	27.59
12 26.26 2.0	793.0	2862.5	27.70
13 26.26 2.0	790.0	2851.6	27.70
14 26.18 2.0	773.5	2839.1	27.24
15 26.22 2.0	89.5	325.8	27.47
16 26.20 2.0	99.5	363.7	27.36
17 26.22 2.0	145.5	529.6	27.47
18 26.20 2.0	170.5	623.2	27.36
19 26.24 2.0	222.5	806.5	27.59
20 26.22 2.0	320.5	1166.5	27.47
21 26.24 2.0	462.5	1676.4	27.59
22 26.22 2.0	579.5	2109.2	27.47
23 26.20 2.0	637.0	2511.0	27.36
24 26.22 2.0	697.0	2536.9	27.47
25 26.22 2.0	760.5	2768.0	27.47
26 26.22 2.0	803.5	2924.5	27.47
27 26.22 2.0	813.0	2959.1	27.47
28 26.26 2.0	748.5	2690.7	27.82
29 26.20 2.0	84.5	308.8	27.36
30 26.22 2.0	89.5	325.8	27.47
31 26.22 2.0	87.5	316.3	27.47
32 26.22 2.0	90.5	326.7	27.70
33 26.24 2.0	96.5	349.8	27.59
34 26.24 2.0	108.0	351.5	27.59
35 26.20 2.0	125.5	458.7	27.36
36 26.26 2.0	162.5	658.8	27.70
37 26.20 2.0	155.5	568.4	27.36
38 26.20 2.0	313.5	1145.8	27.36
39 26.24 2.0	480.0	1739.8	27.59
40 26.22 2.0	572.0	2081.9	27.47
41 26.24 2.0	536.0	1942.8	27.59
42 26.22 2.0	546.0	1987.3	27.47
43 26.20 2.0	81.0	296.1	27.36
44 26.26 2.0	87.0	312.8	27.82
45 26.24 2.0	83.5	302.7	27.59
46 26.24 2.0	89.5	324.4	27.59

2-methyl-20 $\alpha$ -epi 1 $\alpha$ ,25(OH) $_2$ VD $_3$ 誘導体の合成

<Scheme 1>



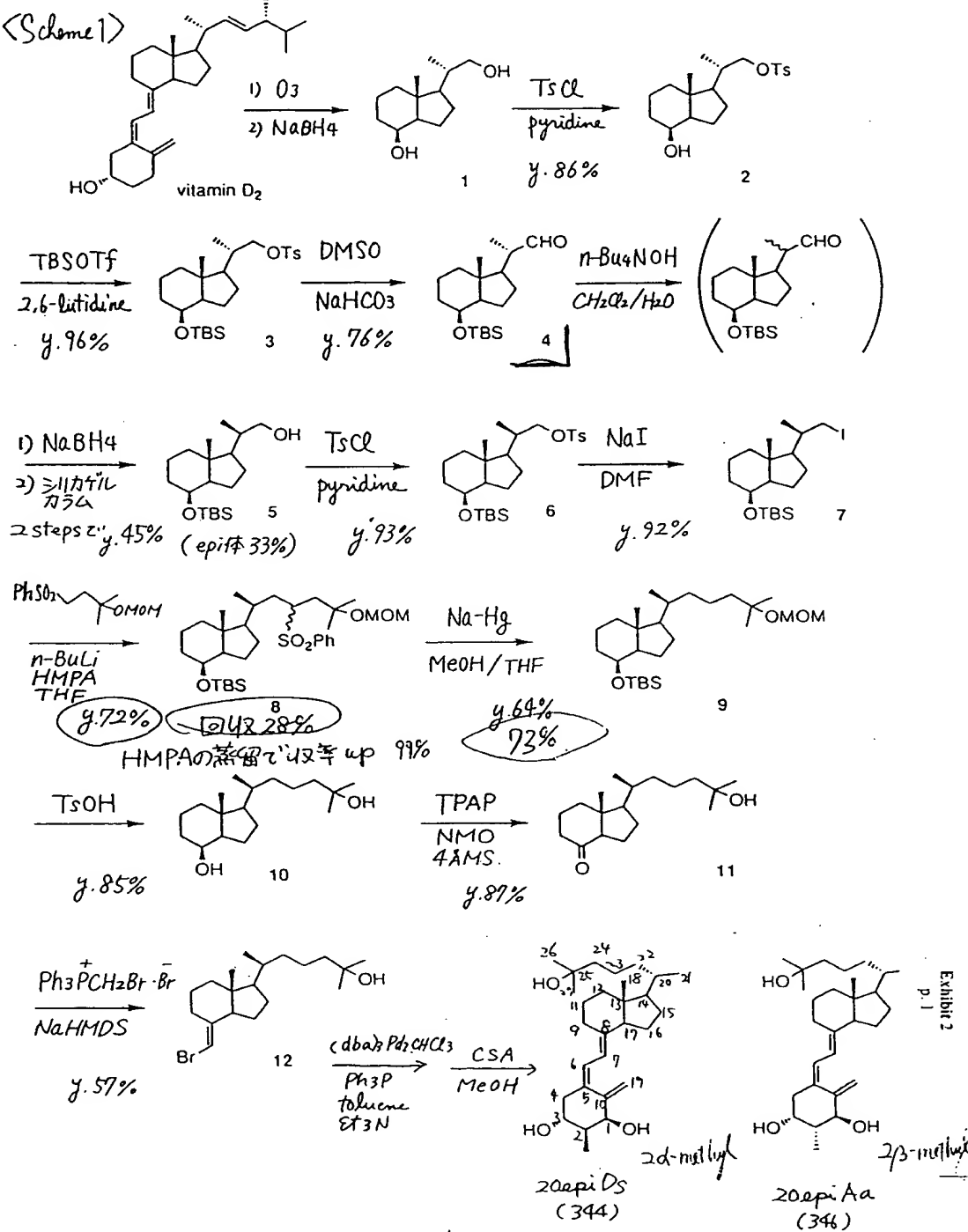


実験セミナー NO. 3

藤島 利江

2-methyl-20 $\alpha$ epi 1 $\alpha$ ,25(OH) $_2$ VD $_3$ 誘導体の合成

<Scheme 1>



# < Bovine Thymus VDR への結合実験 >

① リン酸カリ buffer

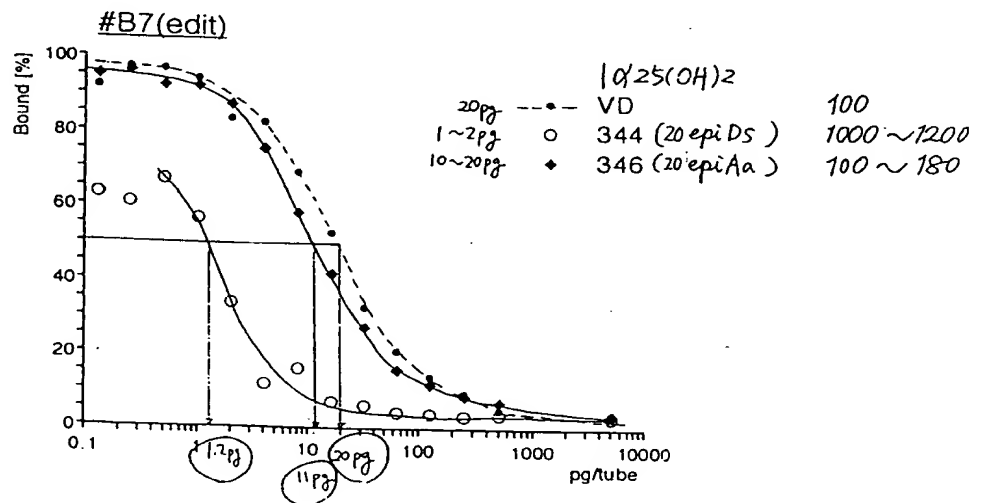
$\left\{ \begin{array}{ll} \text{K}_2\text{HPO}_4 & 0.05\text{M} \\ \text{KH}_2\text{PO}_4 & 0.05\text{M} \\ \text{KCl} & 0.3\text{M} \\ \text{DTT} & 5\text{mM} \end{array} \right. \quad \text{pH } 7.4$

②  $1\alpha,25(\text{OH})_2\text{VD}_3$  #344, #346 を  $\lambda_{\text{max}}$  の  $\epsilon = 18000\epsilon$  用いて濃度調整し希釈系列を作成する。

ウシ胸腺ビタミンDレセプターはヤマサ醤油株式会社より購入し (lot. 110431) 1 アンプル (約 25mg) を 0.05M リン酸 0.5M カリウムバッファー (pH 7.4) 55 ml に溶解した。ビタミンD誘導体のエタノール溶液 50  $\mu\text{l}$  とレセプター溶液 500  $\mu\text{l}$  を室温で1時間ブレインキュベートした後、 $1\alpha,25(\text{OH})_2[^3\text{H}]\text{VD}_3$  溶液 50  $\mu\text{l}$  を最終濃度 0.1nM となるように加えて4℃で一晩インキュベートした。結合と非結合の  $1\alpha,25(\text{OH})_2[^3\text{H}]\text{VD}_3$  はデキストラン-コーテッド-チャコール処理して遠心分離し、上澄に液シンカクテル (ACS-II) を加えて放射活性をカウントした。

freeの drug に対して DCC に かついて 遠心する。

ビタミンD誘導体の活性は 50% 結合阻害する濃度を  $1\alpha,25(\text{OH})_2\text{VD}_3$  を 100 としたときの比で表し評価した。



c.f. 20 epi  $1\alpha,25(\text{OH})_2\text{VD}_3$  の VDR への結合活性

- chicken intestine VDR 120
- bovine thymus VDR 500

7 → 8 (#323)

側鎖部 sulfone 980 mg (3 eq) in dry THF (1.5 ml) と Ar 雰囲気下、  
HMPA 1.5 ml (7 eq) を加え一様とした後、 $-78^{\circ}\text{C}$  に冷却した。  
n-BuLi (1.6 M in n-hexane) 2.3 ml (3 eq) を滴下し  $-78^{\circ}\text{C}$  で  
20 min かくはん後、ヨード体 525 mg (1.20 mmol) in dry THF  
(2 + 洗込み 1 ml) を滴下。  $-78^{\circ}\text{C}$  で 1 hr かくはん後、反応液に  
sat  $\text{NH}_4\text{Cl}$  を加えて EA 抽出。有機層をあわせて brine で洗い、 $\text{MgSO}_4$  上  
脱水。30 $^{\circ}\text{C}$  エバポレート。シリカゲルカラム (EA:n-hex=1:8) にて精製し  
無色 oil 503 mg (y. 72%) を得ると共に 1145 mg の原料を回収 (28%)。

8  $^1\text{H-NMR}$  ( $\text{CDCl}_3/\text{TMS}/400\text{MHz}$ )  $\delta$  -0.02 (3H, s) 0.00 (3H, s)  
0.66 (3H, d,  $J=6.4\text{Hz}$ ) 0.85 & 0.88 (3H, s) 1.23 & 1.27  
(3H, s) 2.32 (1H, dd,  $J=15.3\text{Hz}, 4.3\text{Hz}$ ) 3.26 (1H, m)  
3.30 (3H, s) 3.96 (1H, m) 4.57 (1H, d,  $J=7.3\text{Hz}$ ) 4.67  
(1H, d,  $J=7.3\text{Hz}$ ) 7.55 (2H, t,  $J=6.3\text{Hz}$ ) 7.63 (1H, t,  
 $J=6.3\text{Hz}$ ) 7.88 (2H, d,  $J=6.3\text{Hz}$ )

MS = 580 ( $\text{M}^+$ )

HRMS: calcd for  $\text{C}_{32}\text{H}_{56}\text{O}_5\text{SiS}$  = 580.3620  
found = 580.3618

8 → 9 (#310)

8 165 mg (0.28 mmol) を dry THF 3 ml, dry MeOH 3 ml にとかし  
 $\text{Na}_2\text{HPO}_4$  3.0 g, 5% Na-Hg 9.8 g を加えて Ar 下 10 min かくはん  
overnight。反応液を ether で希釈しセライト 30 $^{\circ}\text{C}$ 。有機層を  
brine で洗い、 $\text{MgSO}_4$  上脱水。30 $^{\circ}\text{C}$  エバポレート。シリカゲル  
カラム (EA:n-hex=1:9) にて精製し 9 無色 oil 80 mg (y. 64%) を  
得ると共に原料 11 mg (7%) を回収。

9  $^1\text{H-NMR}$  ( $\text{CDCl}_3/\text{TMS}/400\text{MHz}$ )  $\delta$  -0.01 (3H, s) 0.01 (3H, s)  
0.81 (3H, d,  $J=6.7\text{Hz}$ ) 0.89 (9H, s) 0.91 (3H, s) 1.21  
(6H, s) 0.98-1.57, 1.64-1.94 (19H, m) 3.36 (3H, s) 3.99  
(1H, m) 4.70 (2H, s)

MS: 440 ( $\text{M}^+$ ), 425 ( $\text{M-Me}$ )<sup>+</sup>

HRMS: calcd for  $\text{C}_{26}\text{H}_{52}\text{O}_3\text{Si}$  = 440.3688  
found = 440.3687



9 → 10 (#316)

ホジ体 9 80mg (0.18 mmol) を MeOH 3 ml に溶解。TsOH·H<sub>2</sub>O 174 mg (0.91 mmol) を加えて rt で 18 hr overnight. 反応液から MeOH をエバポレートし、シリカゲルカラム (EA:n-hex = 1:2) にて精製。無色 oil 43 mg (y. 85%) を得る。

10 <sup>1</sup>H-NMR (CDCl<sub>3</sub>/TMS/400MHz) δ 0.84 (3H, d, J=6.7 Hz) 0.93 (3H, s) 1.21 (6H, s) 4.07 (1H, m)

MS: 264 (M-H<sub>2</sub>O)<sup>+</sup>, 246 (M-2H<sub>2</sub>O)<sup>+</sup>

HRMS: calcd for C<sub>18</sub>H<sub>32</sub>O : 264.2455 (M-H<sub>2</sub>O)  
found : 264.2453

10 → 11 (#326)

アリコ-11 117 mg (0.41 mmol), dry CH<sub>2</sub>Cl<sub>2</sub> (10 ml), 4AMS 30 mg と Ar F rt で 5 分間かくはんする。TPAP 84 mg (0.24 mmol) を加えて 1 hr 20 min 後反応液を small pad of silica gel 上 3 かし。エバポレート。シリカゲルカラム (EA:n-hex = 1:1) にて精製。100 mg (y. 87%) を得る。

11 <sup>1</sup>H-NMR (CDCl<sub>3</sub>/TMS/400MHz) δ 0.64 (3H, s) 0.87 (3H, d, J=6.1 Hz) 1.22 (6H, s) 2.45 (1H, dd, J=11.6 Hz, 7.3 Hz)

MS: 262 (M-H<sub>2</sub>O)<sup>+</sup>

HRMS: calcd for C<sub>18</sub>H<sub>30</sub>O (M-H<sub>2</sub>O) : 262.2298  
found : 262.2297

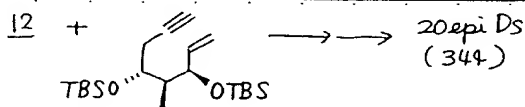
11 → 12 (#334)

(bromomethyl)triphenyl phosphonium bromide 389 mg (5 eq) in dry THF (1.5 ml) と Ar F -60°C に冷却し 1.0 M NaHMDS 0.86 ml (4.8 eq) を加えて -60°C で 1 hr 反応させ。11 50 mg (0.18 mmol) in dry THF (1.5 ml) に transfer する。-60°C → 0°C → rt へと昇温し 1 hr 反応させ。反応液に n-ヘキサンを加えてセライト 3 かし。3 液をエバポレートしてシリカゲルカラム (EA:n-hex = 1:8 → 1:3) にて精製。12 36 mg (y. 56%) の淡黄 oil を得る。

12 <sup>1</sup>H-NMR (CDCl<sub>3</sub>/TMS/CDCl<sub>3</sub>) δ 0.56 (3H, s) 0.85 (3H, d, J=6.4 Hz) 1.22 (6H, s) 2.88 (1H, m) 5.64 (1H, d, J=1.5 Hz)

MS: 356 & 358 (M<sup>+</sup>), 338 & 340 (M-H<sub>2</sub>O)<sup>+</sup>

HRMS: calcd for C<sub>19</sub>H<sub>33</sub>O<sup>79</sup>Br : 356.1716  
found : 356.1715



12 17mg (0.048mmol) を toluene 0.3ml に溶かし Et<sub>3</sub>N 0.45ml を加える。Ar (dba)<sub>3</sub>Pd<sub>2</sub>·CHCl<sub>3</sub> 1.9mg (0.03eq), Ph<sub>3</sub>P 2.5mg (0.3eq) を加え rt で 10min かくはんしつ A 環部 13mg (0.034mmol) を toluene (150μl + 50μl) に加える。赤黒い溶液を rt で 10min かくはんすると黄色溶液になる。120°C の oil bath 上 2.5hr 反応させる反応液を 3か。シヨ+カラム (SiO<sub>2</sub>, EA=n-hex = 1:3) に付し黄色油を得る。(精製せず=次の反応へ。)

ホコ体 MeOH 1ml にとかし CSA 11mg (0.047mmol) を加えて Ar 下 rt で overnight かくはん。MeOH を溜去し水を加え EA 抽出。有機層を 2か。メソ+カラム (SiO<sub>2</sub>, EA=n-hex = 1:1) にて精製。無色結晶 9.3mg (y. 63%) を得る。

#### <HPLCによる精製>

カラム: LiChrosorb RP-18 (7μm), 10×250, No. 301291

溶媒: Acetonitrile = 水 = 70:30

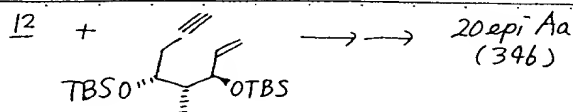
recycler をつて流速 7.0 ml/min

UV (EtOH) :  $\lambda_{max}$  266nm  $\frac{A_{266}}{A_{226}} = 0.57$   
 $\lambda_{min}$  226nm

<sup>1</sup>H-NMR (CDCl<sub>3</sub>-D<sub>2</sub>O/TMS/400MHz)  $\delta$  0.53 (3H, s) 0.85 (3H, d, J=6.7Hz) 1.08 (3H, d, J=6.8Hz) 1.21 (6H, s) 1.12-2.04 (19H, m) 2.23 (1H, dd, J=7.9Hz, 13.4Hz) 2.67 (4.0Hz, 13.4Hz) 2.83 (1H, m) 3.83 (1H, ddd, J=7.9, 4.4, 4.0Hz) 4.29 (1H, d, J=3.3Hz) 5.01 (1H, d, J=1.8Hz) 5.28 (1H, m) 6.01 (1H, d, J=11.3Hz) 6.39 (1H, d, J=11.3Hz)

MS: 430 (M<sup>+</sup>), 412 (M-H<sub>2</sub>O)<sup>+</sup>, 394 (M-2H<sub>2</sub>O)<sup>+</sup>

HRMS: calcd for C<sub>28</sub>H<sub>46</sub>O<sub>3</sub> : 430.3447  
 found : 430.3443



12 15 mg (0.042 mmol) を toluene 0.3 ml に溶かし Et<sub>3</sub>N 0.45 ml を加える (ArF) (dba)<sub>3</sub>Pd<sub>2</sub>CHCl<sub>3</sub> 1.7 mg, Ph<sub>3</sub>P 2.5 mg を加え rt で 10 min かくはん。A 環部 13 mg (0.034 mmol) in toluene (150  $\mu$ l + 50  $\mu$ l) を加え 10 min かくはん。120°C の oil bath 上 4 hr 反応させる。反応液をセライトでろす。ショートカラム (EA:n-hex = 1:3, SiO<sub>2</sub>) に付し。黄色 oil を得る。

ホコリ体と MeOH 1 ml にとかし CSA 11 mg (0.047 mmol) を加えて ArF rt で overnight かくはん。MeOH を溜去し。ろかす。ろ液を加え EA 抽出。有機層を brine で洗い MgSO<sub>4</sub> 上脱水ろす。エバポレート。シリカゲルカラムにて (EA:n-hex = 1:1) 精製後 無色結晶 4.5 mg (y 31%) を得る。

<HPLCによる精製>

20 $\epsilon$ pi Ds と同様の条件

UV (EtOH):  $\lambda_{max}$  263 nm  
 $\lambda_{min}$  228 nm

$$\frac{A_{\lambda_{min}}}{A_{\lambda_{max}}} = 0.55$$

<sup>1</sup>H-NMR (CDCl<sub>3</sub>-D<sub>2</sub>O/TMS/400 MHz)  $\delta$  0.55 (3H, s), 0.85 (3H, d, J = 6.4 Hz), 1.15 (3H, d, J = 6.7 Hz), 1.21 (6H, s), 1.17–2.01 (19H, m), 2.42 (1H, dd, J = 13.9, 4.9 Hz), 2.52 (1H, d, J = 13.9 Hz), 2.82 (1H, dd, J = 11.9 Hz, 4.0 Hz), 3.99–4.04 (1H + 1H, m), 5.02 (1H, t, J = 1.8 Hz), 5.37 (1H, t, J = 1.8 Hz), 6.03 (1H, d, J = 11.3 Hz), 6.35 (1H, d, J = 11.3 Hz)

MS: 430 (M<sup>+</sup>), 412 (M-H<sub>2</sub>O)<sup>+</sup>, 394 (M-2H<sub>2</sub>O)<sup>+</sup>

HRMS: calcd for C<sub>28</sub>H<sub>46</sub>O<sub>3</sub> = 430.3447  
found 430.3441

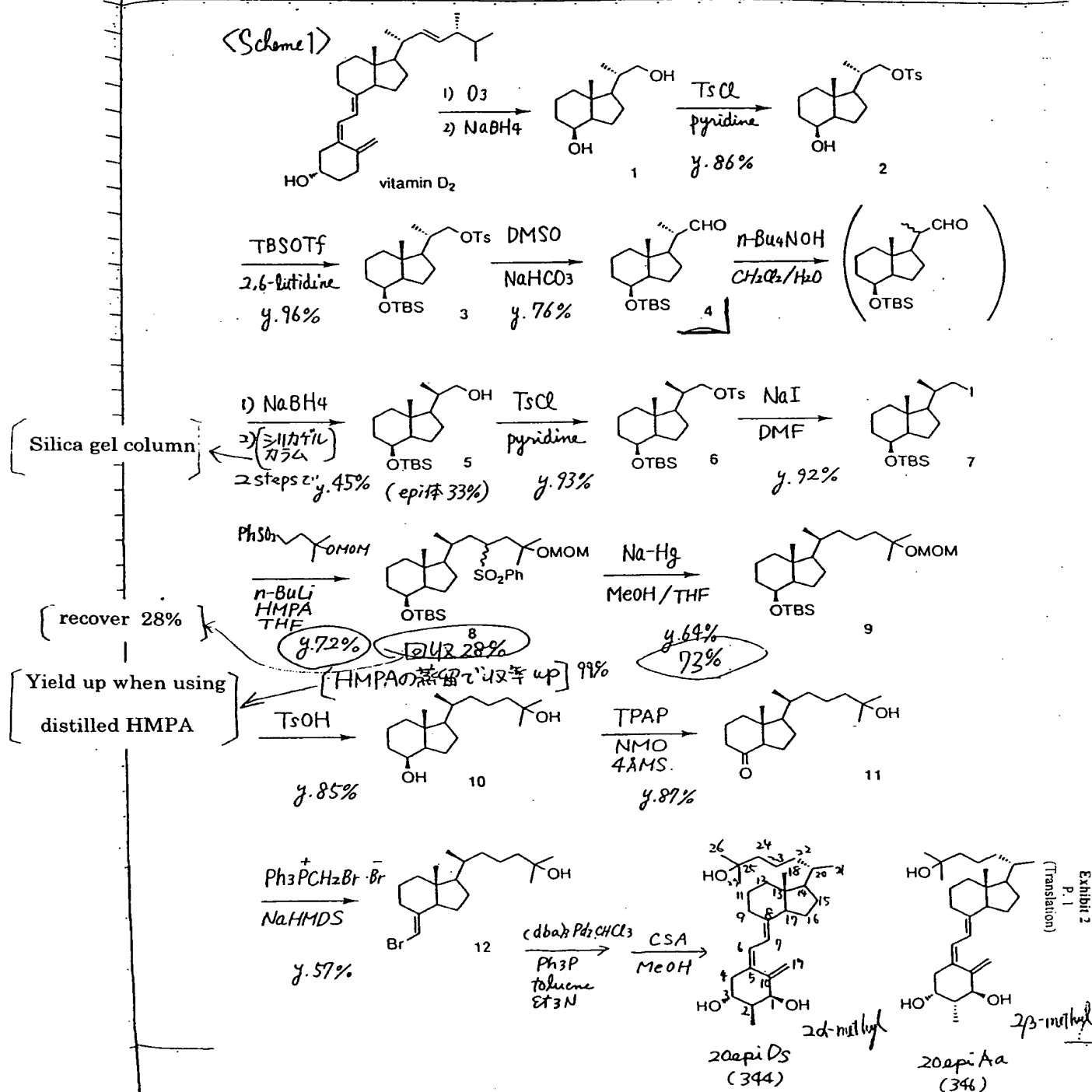
● Synthesis of 2-methyl-20epi 1 $\alpha$ ,25(OH) $_2$ VD $_3$  derivatives

実験セミナー No. 3

藤島 利江

② 2-methyl-20epi 1 $\alpha$ ,25(OH) $_2$ VD $_3$ 誘導体の合成③ 1 $\alpha$ ,25(OH) $_2$ VD $_3$ のA環部分の合成法

&lt;Scheme 1&gt;



<Bovine Thymus VDRへの結合実験>

⑧  $K_{25}(OH)_2VD_3$  #344, #346  $\varepsilon$   $\eta_{max}$  の  $\varepsilon = 18000\varepsilon$  用いて濃度調整し希釈系列を作成する。

free  
- drug  
DCCに  
くっついて  
遠流する

The binding affinity of a compound to be tested for the Vitamin D receptor was expressed by a relative intensity ratio based on 100 for  $1\alpha,25\text{-(OH)}_2[3\text{H}]\text{VD}_3$  by determining the concentration which inhibits the binding of the hot by 50%.

Binding affinity of 20-epi  $1\alpha,25\text{-(OH)}_2\text{VD}_3$  to VDR

7 → 8 (#323)

側鎖部 sulfone 980 mg (3 eq) in dry THF (1.5 ml) を Ar 雰囲気下、  
HMPA 1.5 ml (7 eq) を加え 一樣とし、後、 $-78^{\circ}\text{C}$  に冷却した。  
n-BuLi (1.6 M in n-hexane) 2.3 ml (3 eq) を滴下し  $-78^{\circ}\text{C}$  で  
20 min かくはん後、ヨード体 7 525 mg (1.20 mmol) in dry THF  
(2 + 洗込み 1 ml) を滴下。  $-78^{\circ}\text{C}$  で 1 hr かくはん後、反応液に  
sat.  $\text{NH}_4\text{Cl}$  を加えて EA 抽出。有機層をあわせて brine で洗い、 $\text{MgSO}_4$  上  
脱水。3カ、エバポレート。シリカゲルカラム (EA:n-hex = 1:8) にて精製し  
無色油 8 503 mg (y. 72%) を得ると共に 145 mg の原料 7 を回収 (28%)

Side chain sulfone 980 mg (3 eq) in dry THF (1.5 ml) was added to HMPA 1.5 ml (7 eq) under Ar atmosphere and the mixture was cooled to  $-78^{\circ}\text{C}$  after make the mixture homogeneous. n-BuLi (1.6 M in n-hexane) 2.3 ml (3 eq) was added dropwise to the mixture and stirred for 20 min at  $-78^{\circ}\text{C}$ . Iodo form 7 525 mg (1.20 mmol) in dry THF (2 + rinse 1 ml) was dropwise added to the mixture and stirred for 1 hr at  $-78^{\circ}\text{C}$ . Sat.  $\text{NH}_4\text{Cl}$  was added to the mixture and the resultant mixture was extracted with EA. The extract was combined with organic phase and this solution was washed with brine, dried over  $\text{MgSO}_4$ , filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:8), 503 mg (y. 72%) of colorless oil 8 was obtained with 145 mg of the starting material 7 (28%) was recovered.

8 → 9 (#310)

8 165 mg (0.28 mmol) を dry THF 3 ml, dry MeOH 3 ml と共に  
 $\text{Na}_2\text{HPO}_4$  3.0 g, 5% Na-Hg 9.8 g を加えて Ar 雰囲気下でかくはん  
overnight. 反応液を ether で希釈し、セライト 3カ、有機層を  
brine で洗い、 $\text{MgSO}_4$  上脱水。3カ、エバポレート。シリカゲル  
カラム (EA:n-hex = 1:9) にて精製し 9 無色油 80 mg (y. 64%) を  
得ると共に原料 11 mg (7%) を回収。

8 165 mg (0.28 mmol) was dissolved in dry THF 3 ml and dry MeOH 3 ml,  $\text{Na}_2\text{HPO}_4$  3.0 g, 5% Na-Hg 9.8 g was added to the mixture and stirred overnight under Ar atmosphere at rt. The reaction mixture was diluted with ether and the resultant mixture was filtered through celite. The filtrate organic phase was washed with brine, dried over  $\text{MgSO}_4$ , filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:9), 80 (y. 64%) mg of colorless oil 9 was obtained with 11 mg (7%) of the starting material was recovered.

9 → 10 (#316)

ホコ体 9 80mg (0.18 mmol) を MeOH 3 ml に溶解し、TsOH·H<sub>2</sub>O 174 mg (0.91 mmol) を加えて rt で 18 時間 overnight 反応させた。反応液から MeOH をエバポレートし、シリカゲルカラム (EA:n-hex = 1:2) にて精製。無色 oil 43 mg (y. 85%) を得る。

The protected form 9 80 mg (0.18 mmol) was dissolved in MeOH 3 ml, TsCl·H<sub>2</sub>O 174 mg (0.91 mmol) was added to the mixture and stirred overnight at rt. MeOH was evaporated from the reaction mixture and the residue was purified by silica gel column chromatography (EA:n-hex = 1:2), 43 mg (y. 85%) of colorless oil was obtained.

10 → 11 (#326)

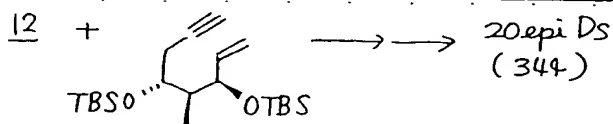
アルコール 10 117mg (0.41 mmol), dry CH<sub>2</sub>Cl<sub>2</sub> (10 ml), 4ÅMS 30 mg と Ar 下 rt で 5 分間攪拌する。TPAP 84 mg (0.24 mmol) を加えて 1 hr 20 min 後反応液を small pad of silica gel 上 3 かし、エバポレート。シリカゲルカラム (EA:n-hex = 1:1) にて精製。100 mg (y. 87%) を得る。

The alcohol 10 117 mg (0.41 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 ml), 4ÅMS 30 mg was added to the mixture and stirred for 5 min under Ar atmosphere at rt. TPAP 84 mg (0.24 mmol) was added to the mixture and the resultant mixture was filtered through small pad of silica gel after 1 hr 20 min. The filtrate was evaporated and the residue was purified by silica gel column chromatography (EA:n-hex = 1:1), 100 mg (y. 87%) was obtained.

11 → 12 (#334)

(bromomethyl)triphenyl phosphonium bromide 389 mg (5 eq) in dry THF (1.5 ml) と Ar 下 -60°C に冷却し 1.0 M NaHMDS 0.86 ml (4.8 eq) を加えて -60°C で 1 hr 反応させた。11 50 mg (0.18 mmol) in dry THF (1.5 ml) に transfer する。-60°C → 0°C → rt へと昇温し 1 hr 反応させた。反応液に n-hexane を加えてセライト 3 かし 3 液をエバポレートしてシリカゲルカラム (EA:n-hex = 1:8 → 1:3) にて精製。12 36 mg (y. 56%) の淡黄色 oil を得る。

(Bromomethyl)triphenyl phosphonium bromide 389 mg (5 eq) in dry THF (1.5 ml) was cooled to -60°C under Ar atmosphere and 1.0 M NaHMDS 0.86 ml (4.8 eq) was added to the mixture. The resultant mixture was reacted for 1 hr at -60°C and the mixture was transferred to 11 50 mg (0.18 mmol) in dry THF (1.5 ml). The reaction mixture was reacted for 1 hr under the reaction temperature was warmed -60°C → 0°C → rt. n-Hexane was added to the reaction mixture and filtered through celite. The filtrate was evaporated and the residue was purified by silica gel column chromatography (EA:n-hex = 1:8 → 1:3), 36 mg (y. 56%) of pale yellow oil 12 was obtained.



12 17mg (0.048mmol) を toluene 0.3ml に溶かし Et<sub>3</sub>N 0.45ml を加える。(Ar) (dba)<sub>3</sub>Pd<sub>2</sub>·CHCl<sub>3</sub> 1.9mg (0.03eq), Ph<sub>3</sub>P 2.5mg (0.3eq) を加え rt でかきはんしつ A 環部 13mg (0.034mmol) in toluene (150μl + 50μl) を加える。赤黒い溶液を rt で 10min かきはんすると黄色溶液になる。120℃の oil bath 上 2.5hr 反応させる。反応液をろか。ショートカラム (SiO<sub>2</sub>, EA:n-hex = 1:3) に付し黄色 oil を得る。(精製せず=次の反応へ。)

ホブ体を MeOH 1ml にとかし CSA 11mg (0.047mmol) を加えて Ar 下 rt で overnight かきはん。MeOH を溜まし水を加え EA 抽出。有機層を あつめて brine で洗う。MgSO<sub>4</sub> 上脱水ろかエバポレート。シリカゲルカラム (EA:n-hex = 1:1) にて精製。無色結晶 9.3mg (y. 63%) を得る。

#### <HPLCによる精製>

カラム: LiChrosorb RP-18 (7μm), 10×250, No. 301291

溶媒: Acetonitrile : 水 = 70 : 30

recycler をつけて 流速 7.0ml/min

12 17 mg (0.048 mmol) was dissolved in toluene 0.3 ml, Et<sub>3</sub>N 0.45 ml was added to the mixture (under Ar atmosphere). (dba)<sub>3</sub>Pd<sub>2</sub>·CHCl<sub>3</sub> 1.9 mg (0.03 eq), Ph<sub>3</sub>P 2.5 mg (0.3 eq) were added to the mixture. A-ring part 13 mg (0.034 mmol) in toluene (150 μl + 50 μl) was added to the mixture under stirring of the mixture at rt. The resultant red-black colored solution was changed to yellow solution during stirring for 10 min at rt. The resultant mixture was reacted for 2.5 hr in an oil bath at 120 °C. The reaction mixture was filtered, the filtrate was evaporated, and the residue was treated with short column chromatography (SiO<sub>2</sub>, EA:n-hex = 1:3), yellow oil was obtained. (The next reaction was carried out without purification)

The protected form was dissolved in MeOH 1.0 ml, CSA 11 mg (0.047 mmol) was added to the mixture, and stirred overnight at rt under Ar atmosphere. MeOH was evaporated, water was added to the resultant residue and extracted with EA. The combined organic phase was washed with brine, dried over MgSO<sub>4</sub>, filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:1), 9.3 mg (y. 63%) of colorless crystal was obtained.

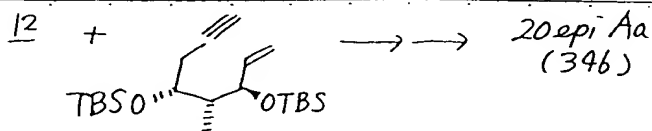
#### <Purification by HPLC>

column: LiChrosorb RP-18 (7 μm), 10 x 250, No. 301291

solvent: Acetonitrile : water = 70 : 30

flow rate 7.0 ml / min with recycler





12 15 mg (0.042 mmol) を toluene 0.3 ml に溶かし Et<sub>3</sub>N 0.45 ml を加える (ArF)  
(dba)<sub>3</sub>Pd<sub>2</sub>·CHCl<sub>3</sub> 1.7 mg, Ph<sub>3</sub>P 2.5 mg を加え rt で 10 min かくはん A 環部  
13 mg (0.034 mmol) in toluene (150 μl + 50 μl) を加え 10 min かくはん. 120 °C の  
oil bath 上 4 hr 反応させる. 反応液をセライトでろし. ショートカラム (EA:n-hex  
= 1:3, SiO<sub>2</sub>) に付し. 黄色油を得る.

ホゴ体と MeOH 1 ml にとかし CSA 11 mg (0.047 mmol) を加えて ArF rt で  
overnight かくはん MeOH を溜去し. 水を加え EA 抽出. 有機層を brine で  
洗い MgSO<sub>4</sub> 上脱水ろす. エバポレート. シリカゲルカラムにて (EA:n-hex = 1:1)  
精製後 無色結晶 4.5 mg (y 31%) を得る.

<HPLCによる精製>

20 epi Ds と同様の条件

12 15 mg (0.042 mmol) was dissolved in toluene 0.3 ml, Et<sub>3</sub>N 0.45 ml was added to the mixture (under Ar atmosphere). (dba)<sub>3</sub>Pd<sub>2</sub>·CHCl<sub>3</sub> 1.7 mg, Ph<sub>3</sub>P 2.5 mg were added to the mixture. A-ring part 13 mg (0.034 mmol) in toluene (150 μl + 50 μl) was added to the mixture under stirring at rt and the mixture was stirred for 10 min. The resultant mixture was reacted for 4 hr in an oil bath at 120 °C. The reaction mixture was filtered through celite, the filtrate was evaporated and the residue was treated with short column chromatography (SiO<sub>2</sub>, EA:n-hex = 1:3), yellow oil was obtained.

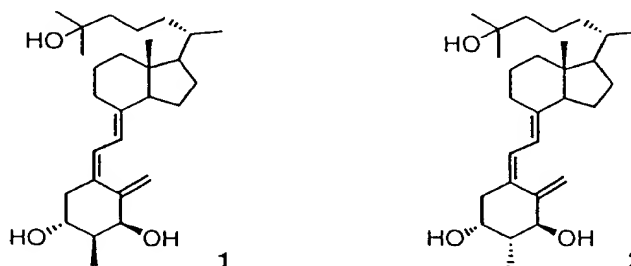
The protected form was dissolved in MeOH 1.0 ml, CSA 11 mg (0.047 mmol) was added to the mixture, and stirred overnight at rt under Ar atmosphere. MeOH was evaporated, water was added to the resultant residue and extracted with EA. The combined organic phase was washed with brine, dried over MgSO<sub>4</sub>, filtrated, and evaporated. The residue was purified by silica gel column chromatography (EA:n-hex = 1:1), 4.5 mg (y. 31%) of colorless crystal was obtained.

<Purification by HPLC>

same condition as 20 epi Ds.

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF 2-METHYL-20-EPI ANALOGUES OF  $1\alpha,25$ -DIHYDROXYVITAMIN  $D_3$ . T. Fujishima, Z.-P. Liu, K. Konno and H. Takayama Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, JAPAN.

Active conformations of the A-ring of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  is still unclear. In order to investigate the conformation-activity relationship of the A-ring portion, we have synthesized the 2-methyl analogues of  $1\alpha,25$ -dihydroxyvitamin  $D_3$ , demonstrating that the introduction of the 2-methyl group elevates the affinity to the nuclear receptor (VDR) in some cases. In the present work, we designed and synthesized 2-methyl-20-epi analogues of  $1\alpha,25$ -dihydroxyvitamin  $D_3$ . The binding affinities of the synthesized compounds were preliminarily tested using the bovine thymus vitamin D receptor. The  $2\alpha$ -methyl-20-epi analogue (1) exhibited about ten-fold higher potency than  $1\alpha,25$ -dihydroxyvitamin  $D_3$ , whereas the  $2\beta$ -methyl-20-epi analogue (2) had similar activity to  $1\alpha,25$ -dihydroxyvitamin  $D_3$ .



# TENTH WORKSHOP ON VITAMIN D -- Strasbourg, France - May 24 - 29, 1997 ABSTRACT REPRODUCTION FORM (Instructions for preparing the abstract are given on the reverse side.)

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☐ (d)  $1,25(OH)_2D_3$  Receptors (biochemistry & molecular biology)  
☐ (e) Gene regulation by vitamin D steroids  
☐ (f) Retinoids &  $1,25(OH)_2D_3$   
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☐ (t) Evolutionary aspects  
☐ (u) Vitamin D-binding protein (DBP)

- ☐ (v) Assay methodology (vitamin D & metabolites)  
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## Clinical Topics and Vitamin D

- ☐ (x)  $1,25(OH)_2D_3$  Receptor (VDR) polymorphisms  
☐ (y) Rickets & osteomalacia  
☐ (z) Osteoporosis  
☐ (za) Renal osteodystrophy  
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☐ (zc) Neonatology/Pregnancy/Development  
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☐ (ze) Dermatology  
☐ (zf) Nutritional aspects  
☐ (zg) Other (clinical topics)

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Signature of submitting author:

Hiroaki Takayama

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2. If using a computer, do not use a font size that is smaller than 10. The font type should be Arial, Helvetica, Roman or Courier; do NOT use Italic or script styles. If you are using an electric typewriter, use ELITE type with a non-smudging ribbon. Only clean corrections will be accepted.
3. Be certain the TITLE IS COMPLETELY CAPITALIZED. The title should be followed by authors' names (initials or first name must precede the last name). Authors' names shall be continuously underlined. Department, institutional affiliation, city, country and postal zip code are required (do not include street address).
4. SINGLE SPACE all typing in the abstract. The text should be a single paragraph which starts with a three-space indentation.
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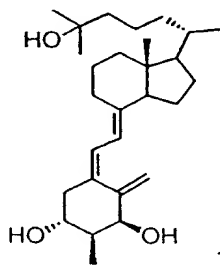
Example:

EXPRESSION OF THE CALBINDIN-D<sub>28K</sub> GENE IS ACC  
CHANGES IN CHROMATIN STRUCTURE. L. Brown and I  
Biochemistry, University of Washington, Seattle, WA, USA 9814

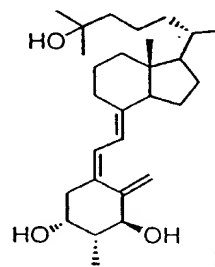
The chromatin structure of the chicken calbindin-D<sub>28K</sub> and flanking DNA was studied in different chicken tissues. Expression of eukaryotic genes is accompanied by changes in the local structural organization of chromatin.

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF 2-METHYL-20-EPI ANALOGUES OF 1 $\alpha$ ,25-DIHYDROXYVITAMIN D<sub>3</sub>. T. Fujishima, Z.-P. Liu, K. Konno and H. Takayama Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, JAPAN.

Active conformation of the A-ring of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> is still unclear. In order to investigate the conformation-activity relationship of the A-ring portion, we have synthesized the 2-methyl analogues of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, demonstrating that the introduction of the 2-methyl group elevates the affinity to the nuclear receptor (VDR) in some cases (preceding abstract). In the present work, we designed and synthesized 2-methyl-20-epi analogues of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. The binding affinities of the synthesized compounds were preliminarily tested using the bovine thymus vitamin D receptor. The 2 $\alpha$ -methyl-20-epi analogue (1) exhibited about ten-fold higher potency than 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, whereas the 2 $\beta$ -methyl-20-epi analogue (2) had similar activity to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Synthesis and biological evaluation of other stereoisomers of the 20-epi analogues will be presented.



1



2

# TENTH WORKSHOP ON VITAMIN D -- Strasbourg, France - May 24 - 29, 1997 ABSTRACT REPRODUCTION FORM

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☐ (c) Vitamin D/Analogues metabolism & catabolism  
☐ (d) 1,25(OH)<sub>2</sub>D<sub>3</sub> Receptors (biochemistry & molecular biology)  
☐ (e) Gene regulation by vitamin D steroids  
☐ (f) Retinoids & 1,25(OH)<sub>2</sub>D<sub>3</sub>  
☐ (g) Rapid/Non-genomic actions  
☐ (h) Cell differentiation/Proliferation  
☐ (i) Transplantation/Immunology  
☐ (j) Brain, neural tissue  
☐ (k) Intestinal & renal actions  
☐ (l) Skin (actions)  
☐ (m) Bone, cartilage  
☐ (n) Muscle  
☐ (o) Calbindins-D and other Ca<sup>2+</sup> binding protein  
☐ (p) Biological actions of vitamin D metabolites (other)  
☐ (q) Development & vitamin D  
☐ (r) Hormone secretion  
☐ (s) Hydroxylases (biochemistry & regulation)  
☐ (t) Evolutionary aspects  
☐ (u) Vitamin D-binding protein (DBP)

- ☐ (v) Assay methodology (vitamin D & metabolites)  
☐ (w) Other (basic science topics)

## Clinical Topics and Vitamin D

- ☐ (x) 1,25(OH)<sub>2</sub>D<sub>3</sub> Receptor (VDR) polymorphisms  
☐ (y) Rickets & osteomalacia  
☐ (z) Osteoporosis  
☐ (za) Renal osteodystrophy  
☐ (zb) Cancer  
☐ (zc) Neonatology/Pregnancy/Development  
☐ (zd) Aging  
☐ (ze) Dermatology  
☐ (zf) Nutritional aspects  
☐ (zg) Other (clinical topics)

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